FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
PU3610USW	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
09/889471	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/01205	19 January 2000	19 January 1999

TITLE OF INVENTION

METHODS OF SCREENING PROTEASE INHIBITORS, OF INDUCING MICE SUSCEPTIBLE TO HIV PROTEASE INHIBITOR-INDUCED DYSLIPIDEMIA, AND GENE ASSOCIATED THEREWITH

APPLICANT(S) FOR DO/EO/US

James Martin LENHARD

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

    Copy of PCT Request 101

Express Mail
EL395942915US

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/889471	PCT/US00/01205	PU3610USW

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☒	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$100.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	142 - 20 =	122	x $18.00	**$2,196.00**
Independent claims	16 - 3 =	13	x $80.00	**$1,040.00**
Multiple Dependent Claims (**check if applicable**).			☐	**$0.00**
	TOTAL OF ABOVE CALCULATIONS		=	**$3,336.00**
☐	Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.			**$0.00**
	SUBTOTAL		=	**$3,336.00**
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).		+	**$0.00**
	TOTAL NATIONAL FEE		=	**$3,336.00**
☐	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (**check if applicable**).			**$0.00**
	TOTAL FEES ENCLOSED		=	**$3,336.00**
			Amount to be: refunded	$
			charged	$

a. ☐ A check in the amount of _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **07-1392** in the amount of **$3,336.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **07-1392** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

23347
PATENT TRADEMARK OFFICE

SIGNATURE

Elizabeth Selby
NAME

38,298
REGISTRATION NUMBER

17 July 2001
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of: James Martin Lenhard
International Application No.: PCT/US00/01205
International Filing Date: 19 January 2000
Title: METHODS OF SCREENING PROTEASE INHIBITORS, OF INDUCING MICE SUSCEPTIBLE TO HIV PROTEASE INHIBITOR-INDUCED DYSLIPIDEMIA, AND GENES ASSOCIATED THEREWITH

Honorable Commissioner of Patents
Washington, D.C. 20231

FIRST PRELIMINARY AMENDMENT

Dear Sir:

The above-identified application is being transmitted herewith for entry in the US National Phase under Chapter II of the PCT. For the purpose of adding the priority information, please amend the application as follows:

In the Abstract:

The Abstract has been placed on a separate sheet of paper according to US practice, as required under 37 CFR 1.72(b).

In the Specification:

On the first line of the specification, after the Title, please add:

--This application is filed pursuant to 35 U.S.C. §371 as a United States National Phase Application of International Application No. PCT/US00/01205 filed 19 January 2000, which claims priority from U.S. Provisional Application Nos. 60/116,300 filed 19 January 1999, 60/137,620 filed 4 June 1999 and 60/146,309 filed 27 July 1999.

IN THE CLAIMS

Please amend the claims as follows:

Please cancel claims 5-25, 37-40, 49-54, 57, 64-70 without prejudice.

Please add the following new claims 71-181:

71. The method of claim 1, wherein the RTA is a protease inhibitor.

72. The method of claim 1, wherein the RTA is a NRTI.

73. The method of claim 1, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

74. The method of claim 73, wherein the receptor ligand is a PPARγ ligand.

75. The method of claim 74, wherein the PPARγ ligand is an agonist of PPARγ.

76. The method of claim 75, wherein the PPARγ agonist is a thiazolidinedione.

77. The method of claim 73 wherein the receptor ligand is a RXR ligand.

78. The method of claim 77, wherein the RXR ligand is an agonist of RXR.

79. The method of claim 78, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

80. The method of claim 73, wherein the receptor ligand is a retinoic acid receptor ligand.

81. The method of claim 80, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

82. The method of claim 73, wherein the receptor ligand is insulin.

83. The method of claim 73, wherein the receptor ligand is an insulin-like growth factor.

84. The method of claim 71, wherein the protease inhibitor is an aspartyl protease inhibitor.

85. The method of claim 84, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

86. The method of claim 85, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

87. The method of claim 72, wherein the NRTI is an HIV NRTI.

88. The method of claim 2, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

89. The method of claim 88, wherein the mesenchymal stem cell is a mammalian primary cell.

90. The method of claim 89, wherein the mammalian primary cell is a human primary cell.

91. The method of claim 3, wherein the cell to which the RTA is administered is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

92. The method of claim 3, wherein the RTA is a protease inhibitor.

93. The method of claim 3, wherein the RTA is a NRTI.

94. The method of claim 3, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

95. The method of claim 94, wherein the receptor ligand is a PPARγ ligand.

96. The method of claim 95, wherein the PPARγ ligand is an agonist of PPARγ.

97. The method of claim 96, wherein the PPARγ agonist is a thiazolidinedione.

98. The method of claim 94, wherein the receptor ligand is a RXR ligand.

99. The method of claim 98, wherein the RXR ligand is an agonist of RXR.

100. The method of claim 99, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

101. The method of claim 94, wherein the receptor ligand is a retinoic acid receptor ligand.

102. The method of claim 101, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

103. The method of claim 94, wherein the receptor ligand is insulin.

104. The method of claim 94, wherein the receptor ligand is an insulin-like growth factor.

105. The method of claim 92, wherein the protease inhibitor is an aspartyl protease inhibitor.

106. The method of claim 105, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

107. The method of claim 106, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

108. The method of claim 93, wherein the NRTI is an HIV NRTI.

109. The method of claim 91, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

110. The method of claim 109, wherein the mesenchymal stem cell is a mammalian primary cell.

111. The method of claim 110, wherein the mammalian primary cell is a human primary cell.

112. The method of claim 4, wherein the cell to which the RTA is administered is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

113. The method of claim 4, wherein the RTA is a protease inhibitor.

114. The method of claim 4, wherein the RTA is a NRTI.

115. The method of claim 4, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

116. The method of claim 115, wherein the receptor ligand is a PPARγ ligand.

117. The method of claim 116, wherein the PPARγ ligand is an agonist of PPARγ.

118. The method of claim 117, wherein the PPARγ agonist is a thiazolidinedione.

119. The method of claim 115, wherein the receptor ligand is a RXR ligand.

120. The method of claim 119, wherein the RXR ligand is an agonist of RXR.

121. The method of claim 120, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

122. The method of claim 115, wherein the receptor ligand is a retinoic acid receptor ligand.

123. The method of claim 122, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

124. The method of claim 115, wherein the receptor ligand is insulin.

125. The method of claim 115, wherein the receptor ligand is an insulin-like growth factor.

126. The method of claim 113, wherein the protease inhibitor is an aspartyl protease inhibitor.

127. The method of claim 126, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

128. The method of claim 127, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

129. The method of claim 114, wherein the NRTI is an HIV NRTI.

130. The method of claim 112, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

131. The method of claim 130, wherein the mesenchymal stem cell is a mammalian primary cell.

132. The method of claim 131, wherein the mammalian primary cell is a human primary cell.

133. The method of claim 35, wherein the compound is screened for potential protease inhibitor activity.

134. The method of claim 35, wherein the receptor ligand is a PPARγ ligand.

135. The method of claim 134 wherein the PPARγ ligand is a thiazolidinedione.

136. The method of claim 134, wherein the ligand is BRL49653.

137. The method of claim 36, wherein the compound is screened for potential protease inhibitor activity.

138. The method of claim 36, wherein the receptor ligand is a PPARγ ligand.

139. The method of claim 138, wherein the PPARγ ligand is a thiazolidinedione.

140. The method of claim 138, wherein the ligand is BRL49653.

141. The method of claim 41, wherein the RTA is a protease inhibitor.

142. The method of claim 41, wherein the mammal is maintained under high-fat diet conditions.

143. The method of claim 41, wherein the mammal is a mouse.

144. The method of claim 143, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

145. The method of claim 43, wherein the RTA is a protease inhibitor.

146. The method of claim 43, wherein the mammal is maintained under high-fat diet conditions.

147. The method of claim 43, wherein the mammal is a mouse.

148. The method of claim 147, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

149. The method of claim 47, wherein the RTA is a protease inhibitor.

150. The method of claim 47, wherein the mammal is maintained under high-fat diet conditions.

151. The method of claim 47, wherein the mammal is a mouse.

152. The method of claim 151, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

153. The method of claim 48, wherein the RTA is a protease inhibitor.

154. The method of claim 48, wherein the mammal is maintained under high-fat diet conditions.

155. The method of claim 48, wherein the mammal is a mouse.

156. The method of claim 155, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

157. The method of claim 48, wherein the retinoid-activated gene is a gene which encodes alkaline phosphatase.

158. The method of claim 48, wherein the retinoid-activated gene is activated by a retinoid nuclear receptor.

159. The transgenic mouse of claim 55, wherein the RTA is a protease inhibitor.

160. The transgenic mouse of claim 56, wherein the RTA is a protease inhibitor.

161. The method of claim 58, wherein the RTA is an HIV protease inhibitor.

162. The method of claim 58, wherein the gene is a retinoid-activated gene.

163. The method of claim 58, wherein the gene is activated by a retinoid nuclear receptor.

164. The method of claim 58, wherein the gene is a PPARγ:RXR-activated gene.

165. The method of claim 58, wherein the gene is a protease inhibitor regulated gene.

166. The method of claim 58, wherein the change in gene expression comprises an increase in gene expression.

167. The method of claim 58, wherein the change in gene expression comprises a decrease in gene expression.

168. The method of claim 60, wherein the RTA is an HIV protease inhibitor.

169. The method of claim 60, wherein the gene is a retinoid-activated gene.

170. The method of claim 60, wherein the gene is activated by a retinoid nuclear receptor.

171. The method of claim 60, wherein the gene is a PPARγ:RXR-activated gene.

172. The method of claim 60, wherein the gene is a protease inhibitor regulated gene.

173. The method of claim 60, wherein the change in gene expression comprises an increase in gene expression.

174. The method of claim 60, wherein the change in gene expression comprises a decrease in gene expression.

175. The method of claim 62, wherein the RTA is an HIV protease inhibitor.

176. The method of claim 62, wherein the gene is a retinoid-activated gene.

177. The method of claim 62, wherein the gene is activated by a retinoid nuclear receptor.

178. The method of claim 62, wherein the gene is a PPARγ:RXR-activated gene.

179. The method of claim 62, wherein the gene is a protease inhibitor regulated gene.

180. The method of claim 62, wherein the change in gene expression comprises an increase in gene expression.

181. The method of claim 62, wherein the change in gene expression comprises a decrease in gene expression.

REMARKS

The above Preliminary Amendment is made to remove multiple dependencies in the claims. No new matter is added by these amendments, and they are fully supported by the specification. A version with markings to show the changes made is attached hereto. Applicants respectfully request the entry of these amendments.

Respectfully submitted,

Date: 17 July 2001

Elizabeth Selby
Attorney of Record, Reg. No. 38,298

GlaxoSmithKline
Corporate Intellectual Property Department
Five Moore Drive, PO Box 13398
Research Triangle Park, NC 27709-3398
Telephone: 919-483-3934
Facsimile: 919-483-7988

CERTIFICATE OF EXPRESS MAILING (37 CFR 1.10)
I hereby certify that this paper (along with any referred to as being attached or enclosed)
is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: Assistant Commissioner of Patents Washington, D.C. 20231 on 17 July 2001

Marilyn Eldridge

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EL395942915US

Version with Markings to Show Changes Made

1. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising

   (a) administering the RTA to a mesenchymal stem cell or pre-adipocyte cell under culture conditions appropriate for adipogenesis; and

   (b) monitoring the cell for an inhibition of adipogenesis;

   whereby inhibition of adipogenesis indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in the patient.

2. The method of claim 1, wherein the RTA is administered to a mesenchymal stem cell.

3. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

   (a) administering the RTA to a cell capable of metabolizing lipids under conditions permissible for lipogenesis; and

   monitoring net lipogenesis in the cell, whereby a change in net lipogenesis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient.

4. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

   (a) administering the RTA to a cell capable of metabolizing lipids under conditions permissible for lipolysis; and

   (b) monitoring net lipolysis in the cell, whereby a change in net lipolysis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient

5. (Cancelled).

6. (Cancelled).

7. (Cancelled).

8. (Cancelled).

9. (Cancelled).

10. (Cancelled).

11. (Cancelled).

12. (Cancelled).

13. (Cancelled).

14. (Cancelled).

15. (Cancelled).

16. (Cancelled).

17. (Cancelled).

18. (Cancelled).

19. (Cancelled).

20. (Cancelled).

21. (Cancelled).

22. (Cancelled).

23. (Cancelled).

24. (Cancelled).

25. (Cancelled).

26. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

(a) administering the RTA to a cell capable of metabolizing lipids under conditions permissible for metabolizing lipids; and

(b) monitoring the expression of a PPARγ:RXR-regulated gene in the cell, whereby a change in gene expression of the PPARγ:RXR-regulated

gene indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient.

27. The method of claim 26, wherein the cell capable of metabolizing lipids is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

28. The method of claim 26, wherein the RTA is a protease inhibitor.

29. The method of claim 26, wherein the PPARγ:RXR-regulated gene is a gene which encodes aP2.

30. The method of claim 26, wherein the PPARγ:RXR-regulated gene is a gene which encodes lipoprotein lipase.

31. A method of screening a PI for its capacity to affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity in a patient comprising:
    (a) administering the PI to a cell containing a retinoid-regulated gene in the presence of a retinoid; and
    (b) monitoring the cell for a change in the expression of the retinoid-activated gene, whereby a change in the expression of the retinoid-activated gene indicates the PI can affect affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity, thereby screening the PI for its capacity to affect affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity in the patient.

32. The method of claim 31, wherein the cell is an adipocyte or a preadipocyte.

33. The method of claim 31, wherein the PI is an HIV PI.

34. The method of claim 31, wherein the retinoid-activated gene is a gene which encodes alkaline phosphatase.

35. A method of screening a compound for its potential to effect fat metabolism comprising:

(a) contacting a PPAR(receptor-ligand complex with the compound; and

(b) monitoring the complex for displacement of the receptor ligand from the complex, whereby a compound that displaces the receptor has a potential to effect fat metabolism, thereby screening the compound for its potential to effect fat metabolism.

36. A method of screening a compound for its potential to effect fat metabolism comprising:

(a) contacting a PPAR(receptor-ligand complex with the compound; and

(b) monitoring the complex for binding of the compound to the complex, whereby a compound that binds to the complex receptor has a potential to effect fat metabolism, thereby screening the compound for its potential to effect fat metabolism.

37. (Cancelled).

38. (Cancelled).

39. (Cancelled).

40. (Cancelled).

41. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising:

(a) administering the RTA to a mammal susceptible to diet-induced obesity; and

(b) monitoring the mammal for an increase in serum lipids, whereby the increase in net serum lipids indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in a patient.

42. The method of claim 41, wherein the change in serum lipids is indicated by a change in serum triglycerides, free fatty acids, glycerol, or cholesterol.

43. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

a) administering the RTA to a mammal susceptible to diet-induced obesity; and

b) monitoring net fat deposition in the mammal, whereby a change in net fat deposition indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient.

44. The method of claim 43, wherein the change in net fat deposition is indicated by a change in the weight of fat pads.

45. The method of claim 43, wherein the change in net fat deposition is indicated by a change in expression or activity of proteins produced by adipocytes.

46. The method of claim 43, wherein the fat deposition results in interscapular or epididymal fat depots.

47. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising:

(a) administering the RTA to a mammal susceptible to diet-induced obesity; and

(b) monitoring the mammal for an increase in blood urea nitrogen or glucose, whereby the increase in blood urea nitrogen or glucose indicates the RTA has the capacity to affect lipodystrophy or dyslipidemia in a patient.

48. A method of screening an RTA for its capacity to affect lipodystrophy, dyslipidemia or retinoid associated toxicities in a patient, comprising:

(a) administering the RTA to a cell containing a retinoid-regulated gene in the presence of a retinoid; and

(a) monitoring the mammal for a change in the expression of a retinoid-activated gene, whereby a change in the expression of the retinoid-activated gene indicates the RTA can affect lipodystrophy, dyslipidemia, or retinoid associated toxicities, thereby screening the RTA for its capacity to affect lipodystrophy, dyslipidemia, or retinoid associated toxicities in a patient.

49. (Cancelled).

50. (Cancelled).

51. (Cancelled).

52. (Cancelled).

53. (Cancelled).

54. (Cancelled).

55. A transgenic mouse whose somatic cells comprise and express a transgene conferring sensitivity to an RTA, wherein the total native and transgene expressed in the transgenic mouse is higher than the native gene expressed in a non-transgenic mouse, which transgenic mouse has a phenotype of increased sensitivity to the RTA.

56. A transgenic mouse whose somatic cells comprise and overexpress ubiquitously in all tissues a transgene conferring sensitivity to an RTA, wherein the total native and transgene expressed in the transgenic mouse is higher than the native gene expressed in a non-transgenic mouse, which transgenic mouse has a phenotype of increased sensitivity to the RTA.

57. (Cancelled).

58. A method of identifying a compound for treating RTA-induced lipodystrophy or

dyslipidemia in a mammal, comprising administering the compound to an RTA-sensitive mouse, and monitoring the mouse for a change in the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to decrease lipodystrophy or dyslipidemia in the mammal and thereby treat RTA-induced lipodystrophy or dyslipidemia in a mammal.

59. The method of claim 58, wherein the RTA is a HIV protease inhibitor, HIV NRTI or HIV NNRTI.

60. A method of detecting a capacity of a compound to cause RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to an RTA-sensitive mouse, monitoring the mouse for a change in expression of a gene and/or the activity of a gene product associated with lipodystrophy, dyslipidemia or retinoid associated toxicities in the mouse, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to cause RTA-induced lipodystrophy, dyslipidemia or retinoid associated toxicities in the mammal.

61. The method of claim 60, wherein the RTA is a protease inhibitor.

62. A method of classifying a patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia, comprising administering RTA to the patient, monitoring the patient for a change in the expression of a gene and/or the activity of a gene associated with lipodystrophy, dyslipidemia or retinoid associated toxicities, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and or the activity of the gene product, an increase in fat distribution, and/or a decrease in serum lipids indicates the patient may be susceptible to lipodystrophy or dyslipidemia; thereby classifying the

patient as being susceptible to RTA-induced lipodystrophy, dyslipidemia or retinoid associated toxicities.

63. The method of claim 62, wherein the RTA is a HIV protease inhibitor, HIV NRTI, HIV, NNRTI.

64. (Cancelled).

65. (Cancelled).

66. (Cancelled).

67. (Cancelled).

68. (Cancelled).

69. (Cancelled).

70. (Cancelled).

71. (New) The method of claim 1, wherein the RTA is a protease inhibitor.

72. (New) The method of claim 1, wherein the RTA is a NRTI.

73. (New) The method of claim 1, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

74. (New) The method of claim 73, wherein the receptor ligand is a PPARγ ligand.

75. (New) The method of claim 74, wherein the PPARγ ligand is an agonist of PPARγ.

76. (New) The method of claim 75, wherein the PPARγ agonist is a thiazolidinedione.

77. (New) The method of claim 73 wherein the receptor ligand is a RXR ligand.

78. (New) The method of claim 77, wherein the RXR ligand is an agonist of RXR.

79. (New) The method of claim 78, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

80. (New) The method of claim 73, wherein the receptor ligand is a retinoic acid receptor ligand.

81. (New) The method of claim 80, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

82. (New) The method of claim 73, wherein the receptor ligand is insulin.

83. (New) The method of claim 73, wherein the receptor ligand is an insulin-like growth factor.

84. (New) The method of claim 71, wherein the protease inhibitor is an aspartyl protease inhibitor.

85. (New) The method of claim 84, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

86. (New) The method of claim 85, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

87. (New) The method of claim 72, wherein the NRTI is an HIV NRTI.

88. (New) The method of claim 2, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

89. (New) The method of claim 88, wherein the mesenchymal stem cell is a mammalian primary cell.

90. (New) The method of claim 89, wherein the mammalian primary cell is a human primary cell.

91. (New) The method of claim 3, wherein the cell to which the RTA is administered is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

92. (New) The method of claim 3, wherein the RTA is a protease inhibitor.

93. (New) The method of claim 3, wherein the RTA is a NRTI.

94. (New) The method of claim 3, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

95. (New) The method of claim 94, wherein the receptor ligand is a PPARγ ligand.

96. (New) The method of claim 95, wherein the PPARγ ligand is an agonist of PPARγ.

97. (New) The method of claim 96, wherein the PPARγ agonist is a thiazolidinedione.

98. (New) The method of claim 94, wherein the receptor ligand is a RXR ligand.

99. (New) The method of claim 98, wherein the RXR ligand is an agonist of RXR.

100. (New) The method of claim 99, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

101. (New) The method of claim 94, wherein the receptor ligand is a retinoic acid receptor ligand.

102. (New) The method of claim 101, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

103. (New) The method of claim 94, wherein the receptor ligand is insulin.

104. (New) The method of claim 94, wherein the receptor ligand is an insulin-like growth factor.

105. (New) The method of claim 92, wherein the protease inhibitor is an aspartyl protease inhibitor.

106. (New) The method of claim 105, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

107. (New) The method of claim 106, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

108. (New) The method of claim 93, wherein the NRTI is an HIV NRTI.

109. (New) The method of claim 91, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

110. (New) The method of claim 109, wherein the mesenchymal stem cell is a mammalian primary cell.

111. (New) The method of claim 110, wherein the mammalian primary cell is a human primary cell.

112. (New) The method of claim 4, wherein the cell to which the RTA is administered is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

113. (New) The method of claim 4, wherein the RTA is a protease inhibitor.

114. (New) The method of claim 4, wherein the RTA is a NRTI.

115. (New) The method of claim 4, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

116. (New) The method of claim 115, wherein the receptor ligand is a PPARγ ligand.

117. (New) The method of claim 116, wherein the PPARγ ligand is an agonist of PPARγ.

118. (New) The method of claim 117, wherein the PPARγ agonist is a thiazolidinedione.

119. (New) The method of claim 115, wherein the receptor ligand is a RXR ligand.

120. (New) The method of claim 119, wherein the RXR ligand is an agonist of RXR.

121. (New) The method of claim 120, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

122. (New) The method of claim 115, wherein the receptor ligand is a retinoic acid receptor ligand.

123. (New) The method of claim 122, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

124. (New) The method of claim 115, wherein the receptor ligand is insulin.

125. (New) The method of claim 115, wherein the receptor ligand is an insulin-like growth factor.

126. (New) The method of claim 113, wherein the protease inhibitor is an aspartyl protease inhibitor.

127. (New) The method of claim 126, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

128. (New) The method of claim 127, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

129. (New) The method of claim 114, wherein the NRTI is an HIV NRTI.

130. (New) The method of claim 112, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

131. (New) The method of claim 130, wherein the mesenchymal stem cell is a mammalian primary cell.

132. (New) The method of claim 131, wherein the mammalian primary cell is a human primary cell.

133. (New) The method of claim 35, wherein the compound is screened for potential protease inhibitor activity.

134. (New) The method of claim 35, wherein the receptor ligand is a PPARγ ligand.

135. (New) The method of claim 134 wherein the PPARγ ligand is a thiazolidinedione.

136. (New) The method of claim 134, wherein the ligand is BRL49653.

137. (New) The method of claim 36, wherein the compound is screened for potential protease inhibitor activity.

138. (New) The method of claim 36, wherein the receptor ligand is a PPARγ ligand.

139. (New) The method of claim 138, wherein the PPARγ ligand is a thiazolidinedione.

140. (New) The method of claim 138, wherein the ligand is BRL49653.

141. (New) The method of claim 41, wherein the RTA is a protease inhibitor.

142. (New) The method of claim 41, wherein the mammal is maintained under high-fat diet conditions.

143. (New) The method of claim 41, wherein the mammal is a mouse.

144. (New) The method of claim 143, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

145. (New) The method of claim 43, wherein the RTA is a protease inhibitor.

146. (New) The method of claim 43, wherein the mammal is maintained under high-fat diet conditions.

147. (New) The method of claim 43, wherein the mammal is a mouse.

148. (New) The method of claim 147, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

149. (New) The method of claim 47, wherein the RTA is a protease inhibitor.

150. (New) The method of claim 47, wherein the mammal is maintained under high-fat diet conditions.

151. (New) The method of claim 47, wherein the mammal is a mouse.

152. (New) The method of claim 151, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

153. (New) The method of claim 48, wherein the RTA is a protease inhibitor.

154. (New) The method of claim 48, wherein the mammal is maintained under high-fat diet conditions.

155. (New) The method of claim 48, wherein the mammal is a mouse.

156. (New) The method of claim 155, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

157. (New) The method of claim 48, wherein the retinoid-activated gene is a gene which encodes alkaline phosphatase.

158. (New) The method of claim 48, wherein the retinoid-activated gene is activated by a retinoid nuclear receptor.

159. (New) The transgenic mouse of claim 55, wherein the RTA is a protease inhibitor.

160. (New) The transgenic mouse of claim 56, wherein the RTA is a protease inhibitor.

161. (New) The method of claim 58, wherein the RTA is an HIV protease inhibitor.

162. (New) The method of claim 58, wherein the gene is a retinoid-activated gene.

163. (New) The method of claim 58, wherein the gene is activated by a retinoid nuclear receptor.

164. (New) The method of claim 58, wherein the gene is a PPARγ:RXR-activated gene.

165. (New) The method of claim 58, wherein the gene is a protease inhibitor regulated gene.

166. (New) The method of claim 58, wherein the change in gene expression comprises an increase in gene expression.

167. (New) The method of claim 58, wherein the change in gene expression comprises a decrease in gene expression.

168. (New) The method of claim 60, wherein the RTA is an HIV protease inhibitor.

169. (New) The method of claim 60, wherein the gene is a retinoid-activated gene.

170. (New) The method of claim 60, wherein the gene is activated by a retinoid

nuclear receptor.

171. (New) The method of claim 60, wherein the gene is a PPARγ:RXR-activated gene.

172. (New) The method of claim 60, wherein the gene is a protease inhibitor regulated gene.

173. (New) The method of claim 60, wherein the change in gene expression comprises an increase in gene expression.

174. (New) The method of claim 60, wherein the change in gene expression comprises a decrease in gene expression.

175. (New) The method of claim 62, wherein the RTA is an HIV protease inhibitor.

176. (New) The method of claim 62, wherein the gene is a retinoid-activated gene.

177. (New) The method of claim 62, wherein the gene is activated by a retinoid nuclear receptor.

178. (New) The method of claim 62, wherein the gene is a PPARγ:RXR-activated gene.

179. (New) The method of claim 62, wherein the gene is a protease inhibitor regulated gene.

180. (New) The method of claim 62, wherein the change in gene expression comprises an increase in gene expression.

181. (New) The method of claim 62, wherein the change in gene expression comprises a decrease in gene expression.

ABSTRACT

METHODS OF SCREENING PROTEASE INHIBITORS, OF INDUCING MICE SUSCEPTIBLE TO HIV PROTEASE INHIBITOR-INDUCED DYSLIPIDEMIA, AND GENES ASSOCIATED THEREWITH

The present invention relates generally to the side effects caused by retroviral therapies, including protease inhibitors, nucleoside reverse transcriptase inhibitors, and non-nucleosidereverse transcriptase inhibitors. Specifically, the present invention provides methods of screening a protease inhibitor for its capacity to affect symptoms or clinical conditions associated with lipodystrophy or dyslipidemia and related metabolic disorders, such as metabolic syndrome X, obesity, cardiovascular disorders, and impaired glucose tolerance in diabetes, in a patient.

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BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to the side effects caused by retroviral therapies, including protease inhibitors, nucleoside reverse transcriptase inhibitors, and non-nucleoside reverse transcriptase inhibitors. Specifically, the present invention provides methods of screening a protease inhibitor for its capacity to affect symptoms or clinical conditions associated with lipodystrophy or dyslipidemia and related metabolic disorders, such as metabolic syndrome X, obesity, cardiovascular disorders, and impaired glucose tolerance in diabetes, in a patient.

BACKGROUND ART

Highly active antiretroviral therapy ("HAART") is currently the preferred treatment for AIDS patients. It involves the combined use of nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are antiretroviral drugs used in therapeutic treatment of AIDS patients. Both classes of drugs inhibit HIV reverse transcriptase (RT) and suppress replication of the virus.

NRTIs are dideoxy dNTP analogs that interact with HIV RT and compete with dNTPs during DNA synthesis. These drugs resemble natural nucleotide bases, are phosphorylated by intracellular enzymes to nucleosidetriphosphates, interact directly with the HIV RT substrate binding site and are incorporated into newly synthesized DNA. Examples of NRTIs include AZT, 3TC, abacavir/ABC, D4T and DDI.

The NNRTIs interact with the RT at an allosterically located site spatially close to the nucleoside binding site. A cooperative interaction between the two sites increases their inhibitory effect on HIV RT, and thus combination therapy with NNRTIs and NRTIs is an effective treatment. Examples of NNRTIs include efavirenz (EFV), nevirapine (NVP) and delavirdine (DLV).

HIV aspartyl protease inhibitors (PIs), such as saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir, are approved for AIDS therapy. These inhibitors show multiple beneficial effects in the clinic, including reduced viral load and opportunistic infections, and increased CD4' T-lymphocyte numbers and patient well being (*Lea et al.* (1996) Drugs 52, 541-8 and *Jarvis et al.* (1998) Drugs 56, 147-67).

The use of multiple drugs in HAART therapy overcomes the ability of the virus to become resistant by mutation. Unfortunately, the therapy is associated with unusual adverse affects, including peripheral fat wasting (lipodystrophy), changes in adipose tissue mass and distribution such as in the development of cervical or visceral fat pads, hypertriglyceridemia, and a condition known as metabolic syndrome X, which comprises obesity, non-insulin-dependent diabetes melitus, hypertension, and dyslipidemia. (*Carr et al.* (1998) AIDS 12, F51-F58, *Walli et al.* (1998) AIDS 12, F167-73, *Landsberg et al.* (1996) Hypertens. Res. Jun;19 Suppl 1:S51-5, and *Minchoff et al.* (1996) Nurse Pract. Jun;21(6):74-5, 79-80, 83-6).

For example, by May of 1997, there were 83 reports to the FDA of new or exacerbated cases of diabetes or hyperglycemia (high blood sugar) in HIV-infected patients who were receiving protease inhibitor therapy. By November of that same year, the number of reports had increased to 230. Of the 83 original cases, 27 required hospitalization, including six that were life threatening. Average time to onset was 76 days after initiating protease inhibitor treatment, but in some reports symptoms appear in as little as four days. Five cases resulted in ketoacidosis, a serious diabetes-related condition that is characterized by a fruity mouth odor, nausea, vomiting, dehydration, weight loss, and if untreated, coma or death. The initial 83 reports led the FDA to issue a Public Health Advisory in June of 1997 (GMHC Treatment Issues, Volume 12,

Number 1, Winter, 1997/1998). One of the more unusual symptoms experienced by people taking protease inhibitors were growths of atypical fat-like tissues in the stomach (popularly called "Crix belly") and upper back ("buffalo humps"). Other patients experience significant breast enlargement. These abnormal fat deposits can be accompanied by loss of mass and strength in the limbs and buttocks. Some patients describe muscle wasting in the arms, chest and legs as well as fatty growths in the upper back and neck area that increased in size after starting protease inhibitor therapy with indinavir, the condition known as "lipodystrophy" (an abnormality in the distribution of fats in the body). Indinavir may also cause changes in fat metabolism which result in dry skin, hair loss, and nail loss. (Lenhard et al., Biochemical Pharmacology, *In Press*.)

Since that time, there has still not been developed an effective method of determining which of the current and/or potential retroviral therapeutic agents (RTAs)which include PIs, NRTIs, and NNRTIs, have the capacity to lead to these side effects and which do not. Therefore there is a clear need for such methods to be developed.

The cause of these serious side effects is unknown, and two prominent groups studying this phenomenon have reached contradictory conclusions which exemplifies the confusion in the art and the lack of direction for future research. (*Carr et al.* and *Gagnon et al.* (1998) The Lancet 352, 1032). Specifically, *Carr et al.* speculate that PIs inhibit adipogenesis by blocking the synthesis of cis-9-retinoic acid from all-trans retinoic acid, which is disproved by the experiments disclosed in the Example contained herein. *Gagnon et al.* state that PIs actually enhance adipogenesis, which not only is in contradiction with the art which teaches that PIs can cause perpherial fat wasting, but is admittedly ". . . at odds with a hypothesis by Carr and colleagues. . . ." (page 1032). Furthermore, since not all the PIs result in the same side effects, there is a need for a method to distinguish between those PIs that cause the undesirable side effects and those that do not. One approach to solving this problem can be to monitor

the effect the PIs, as well as NRTIs and NNRTIs, have on adipogenesis, lipogenesis, and lipolysis.

Alterations in the size and number of adipocytes may contribute to changes in adipose tissue mass and distribution, which then may result in changes in lipid and carbohydrate metabolism. (*Shimomura et al.* (1998) Genes Dev. 12, 3182-94 and *Geloen et al.* (1989) Am. J. Physiol. 257, E547-53). Adipocyte size increases after treatment with agents such as insulin, which stimulates triglyceride synthesis (lipogenesis) and inhibits lipid catabolism (lipolysis). (*Krotkiewski et al.* (1976) Acta Physiol. Scand. 96, 122-7). Adipocyte numbers are regulated by agents that affect differentiation of preadipocyte cells into adipocytes (adipogenesis) (*Martin et al.* (1998) Proc. Soc. Exp. Biol. Med. 219, 200-10) and cell death (necrosis).

Adipogenesis is partially controlled by two nuclear receptors, termed peroxisome proliferator activated receptor γ (PPARγ) and retinoid X receptor γ (RXRγ) (*Tontonoz et al.* (1994) Genes Dev. 8, 1224-34.). Agonists for PPARγ and RXRγ include the thiazolidinediones (e.g., BRL49653) and rexinoids (e.g., LGD1069), respectively (*Lehmann et al.* (1995) J. Biol. Chem. 270, 12953-6 and *Boehm et al.* (1994) J. Med. Chem. 37, 2930-41). Thiazolidinediones, rexinoids, and insulin stimulate adipogenesis *in vitro* (*Martin et al.*, *Tontonoz et al.*, *Kletzien et al.* (1992) Mol. Pharmacol. 41, 393-8, and *Schulman et al.* (1998) Mol. Cell Biol. 18, 3483-94) and markedly improve glucose utilization *in vivo*, indicating adipocytes, in part, mediate the anti-diabetic effects of these agents. As PI therapy is associated with peripheral fat wasting and diabetes (*Carr et al.* and *Walli et al.*), it is important to determine if PIs have an effect on adipocyte metabolism and PPARγ/RXR signaling pathways *in vitro*. Similarly, as PIs, NRTIs, and NNRTIs are preferably used in combination in a therapeutic protocol for treatment of HIV positive patients, it is important to determine the effect of NRTIs and NNRTIs on adipocyte metabolism and PPARγ/RXR signaling pathways *in vitro*. Additionally, since there are multiple parameters which may affect metabolism and lead to the deleterious clinical effects,

there is a need in the art for a means for detecting changes in these factors regulating fat metabolism and adipogenesis which result from the administration of RTAs.

Further, there is currently no accepted *in vivo* or animal model for predicting whether an RTA or potential RTA can lead to lipodystrophy or dyslipidemia. To date, such an animal model has not been developed and potential side-effects of RTAs may not be observed until the drug is actually administered to human patients. For example, *Ye et al.*, report ritonavir decreases serum triglycerides in rodents. In contrast, PIs elevate triglycerides in humans. (*Ye et al.* AIDS 1998 Nov 12;12(16):2236-7). It was speculated by *Ye et al.*, that this paradox may be due to species-specific effects of PIs on lipid metabolism. These observations indicate that rodents would not be a good model for predicting the ability of PIs to cause lipodystrophy or dyslipidemia in patients. Therefore there is a clear need to develop an animal model so that one can predict whether current or potential RTAscan lead to these adverse side-effects.

This invention therefore fulfills both of these needs by providing novel methods to distinguish those RTAs that have the capacity to stimulate deleterious clinical side effects such as lipodystrophy from those that do not. Additionally, methods are provided herein which monitor molecular mechanisms and reactions which are highly correlated with threshold events in adipogenesis, lipogenesis, and lipolysis. These methods are highly sensitive and can be used to detect cellular changes that can otherwise be undetected. This represents a major advantage in the art since the methods of the prior art do not address molecular mechanisms regulating fat metabolism. Furthermore, the prior art methods demonstrate contradictory results, are relatively insensitive, and are based on broad phenotypic markers that are evaluated subjectively, and therefore are potentially inconsistent from test to test. Finally, the current invention discloses an animal model for predicting whether RTAs can lead to altered fat metabolism, and therefore whether the administration of the RTAscan lead to adverse side-effects associated with altered fat metabolism. In contrast to the results of *Ye et al.*, the current invention indicates rodents can be used to screen an RTA for its capacity to affect lipodystrophy or dyslipidemia in patients. In particular, when

animals susceptible to diet-induced obesity are fed a standard high-fat diet and treated with PIs, they develop symptoms similar to those observed in humans treated with PIs. The data presented within this invention is the first to indicate both environmental (high fat diet) and genetic (susceptibility to obesity) factors predispose mammals to adverse reactions caused by PIs.

SUMMARY OF THE INVENTION

The present invention provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising administering the RTA to a mesenchymal stem cell or pre-adipocyte cell under culture conditions appropriate for adipogenesis, and monitoring the cell for an inhibition of adipogenesis, whereby inhibition of adipogenesis indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in the patient.

Further provided by this invention is a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to cell capable of metabolizing lipids under conditions permissible for lipogenesis, and monitoring net lipogenesis in the cell, whereby a decrease in net lipogenesis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient.

In another aspect, the invention is a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to cell capable of metabolizing lipids under conditions permissible lipolysis, and monitoring net lipolysis in the cell, whereby an increase in net lipolysis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient

In another aspect, the invention is a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering

the RTA to a cell capable of producing or metabolizing lipids under conditions permissible for metabolizing or producing lipids, and monitoring the expression of a PPARγ:RXR-regulated gene in the cell, whereby a change in gene expression of the PPARγ:RXR-regulated gene indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient.

In another aspect, the invention is A method of screening an RTA for its capacity to affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity in a patient comprising administering the RTA to a cell containing a retinoid-regulated gene in the presence of a retinoid, and monitoring the cell for a change in the expression of the retinoid-activated gene, whereby a change in the expression of the retinoid-activated gene indicates the RTA can affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity, thereby screening the RTA for its capacity to lipodystrophy, dyslipidemia, or retinoid-associated toxicity in the patient.

In another aspect, the invention provides a method of screening a compound for potential RTA activity comprising contacting a PPARγ receptor-ligand complex with the compound, and monitoring the complex for displacement of the ligand from the complex, whereby a compound that displaces the ligand is a potential RTA, thereby screening the compound for its potential RTA activity.

In yet another aspect, the invention provides a method of screening a compound for potential RTA activity comprising contacting a PPARγ receptor-ligand complex with the compound and monitoring the complex for binding of the compound to the complex, whereby a compound that binds to the complex is a potential RTA, thereby screening the compound for its RTA activity.

In yet another embodiment, the invention provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring the mammal for an increase in serum lipids, whereby the increase in net

serum lipids indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in a patient.

The invention also provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring net fat deposition in the mammal, whereby a change in net fat deposition indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient.

The invention also provides a method of identifying a compound for treating RTA-induced lipodystrophy or dyslipidemia in a patient, comprising administering the compound to an RTA-sensitive mouse, and monitoring the mouse for a change in the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to decrease lipodystrophy or dyslipidemia in the mammal and thereby treat RTA-induced lipodystrophy or dyslipidemia in the patient.

In another embodiment, the invention provides a method of detecting a capacity of a compound to cause RTA-induced lipodystrophy or dyslipidemia in a patient, comprising administering the compound to an RTA-sensitive mouse, monitoring the mouse for a change in expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia in the mouse, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to cause RTA-induced lipodystrophy or dyslipidemia in the patient.

The invention also provides a method of classifying a patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia, comprising administering RTA to the patient, monitoring the patient for a change in the expression of a gene

and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and or the activity of the gene product, an increase in fat distribution, and/or a decrease in serum lipids indicates the patient may be susceptible to RTA-induced lipodystrophy or dyslipidemia; thereby classifying the patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia.

Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the effects of protease inhibitors on lipolysis using stem cells. C3H10T1/2 stem cells were cultured in 96-well microtiter plates (12×10^3 cells/cm^2) and treated with 1 μM BRL49653, 1 μM LGD1069, and 1 μM insulin (conditions which suppress lipolysis). At the same time, vehicle (dimethylsulfoxide, DMSO) or test compounds were suspended to 40 mM in DMSO and serially diluted from 40 μM to < 40 nM into the culture medium of the preadipocyte stem cells. The cells were cultured for 7 days and lipolysis was measured as described in *Lenhard et al.* (Biochem. Pharmacol. 54:801-808 (1997).

Figure 2 shows the effects of protease inhibitors on lipolysis using adipocytes with and without protein synthesis. Adipocytes were prepared from C3H10T1/2 clone 8 murine fibroblasts (ATCC) as described in *Paulik et al.* (1997) Cell Tissue Res.

290:79-87. Isoproterenol, an andrenergic agonist that stimulates lipolysis, was used as a positive control.

Figure 3 shows a the effects of protease inhibitors total triglyceride accumulation in differentiating preadipocytes. One day after passage of C3H10T1/2 cells into 96-well microtiter plates (12.5 x 10^3 cells/cm^2), the cells were treated with 1 μM BRL49653, 1 μM LGD1069, and 1 μM insulin (conditions which stimulate triglyceride accumulation). At the same time, test compounds were suspended to 40 nM in DMSO and serially diluted from 40 μM to < 40 nM into the culture medium of the preadipocyte stem cells. The cells were cultured for 7 days and the accumulation of triglycerides was measured as described in *Lenhard et al.* (Biochem. Pharmacol. 54:801-808 (1997).

Figure 4 shows the effects of protease inhibitors on lipogenesis using adipocyte cells. Adipocytes were prepared as described in *Paulik et al.* (1997) Cell Tissue Res. 290:79-87. Mature adipocytes were treated for three days with the indicated compounds (20 μM) and lipogenesis was measured as described in *Lenhard et al* (1997).

Figure 5 shows the effects of protease inhibitors on aP2 and LPL gene expression. One day after passage into 96-well microtiter plates (12.5 x 10^3 cells/cm^2), C3H10T1/2 cells were treated with 1 μM BRL49653, 1 μM LGD1069, and 1 μM insulin (conditions which activate endogenous PPARγ). At the same time, cells were treated with 20 μM of test compounds or vehicle (dimethylsulfoxide, DMSO). The cells were cultured for 7 days and total RNA was isolated using using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Ten μg of RNA was electrophoresed in agarose gels and transferred to nitrocellulose. The blot was probed with mouse aP2 and lipoprotein lipase (LPL) probes labeled via the random-priming technique (Prime-It II Kit, Stratagene, La Jolla, CA) with [α^{32}P]dCTP. The autoradigraphs were analysed with a Biorad Imaging System.

Figure 6 shows the effects of protease inhibitors on the activity of alkaline phosphatase in C3H10T1/2 cells. C3H10T1/2 preadipocytes were cultured for seven

days in the presence of various PIs (amprenavir, nelfinavir, ritonavir, saquinavir, or indinavir) and retinoids (cis 9-retinoic acid, ATRA, AGN 193109, or CH55). Subsequently, ALP activity was measured as described in *Paulik et al.*

Figure 7 shows the effects of protease inhibitors on the activity of alkaline phosphatase in C3Hl0T1/2 cells in the presence of 100 nM ATRA, but not cis 9-retinoic acid. C3H10T1/2 preadipocytes were cultured for seven days in the presence of various PIs (amprenavir, nelfinavir, ritonavir, saquinavir, or indinavir) and 100 nM ATRA. Subsequently, ALP activity was measured as described in *Paulik et al.*

Figure 8 shows the effects of protease inhibitors on the activity of alkaline phosphatase in C3Hl0T1/2 cells in the presence of CH55. C3H10T1/2 preadipocytes were cultured for seven days in the presence of various PIs (amprenavir, nelfinavir, ritonavir, saquinavir, or indinavir) and CH55. Subsequently, ALP activity was measured as described in *Paulik et al.*

Figure 9 shows the effects of saquinavir on the binding of BRL49653 to PPARγ. Test compounds were assayed for competitive-inhibition of BRL49653 binding to human PPARγ ligand-binding domain as described in *Nichols et al.* (1998) Anal. Biochem. 257:112-119.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Example included therein.

Before the present methods are disclosed and described, it is to be understood that this invention is not limited to specific compounds and methods, as such may of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, a cell can mean a single cell or more than one cell.

As used herein, a "retroviral therapeutic agent" (RTA) refers to any compound which inhibits, reduces or otherwise adversely interferes with a retroviral infection, such as a compound which may be classified as a protease inhibitor, NRTI, or NNRTI. In a preferred embodiment, the RTA will be useful for the treatment of HIV-positive patients. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI. .

The present invention provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising administering the RTA to a mesenchymal stem cell or pre-adipocyte cell under culture conditions appropriate for adipogenesis, and monitoring the cell for an inhibition of adipogenesis, whereby inhibition of adipogenesis indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in the patient. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

Further provided by this invention is a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to cell capable of producing lipids under conditions permissible for lipogenesis, and

monitoring net lipogenesis in the cell, whereby a decrease in net lipogenesis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In another aspect, the invention is a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to cell capable of metabolizing lipids under conditions permissible lipolysis, and monitoring net lipolysis in the cell, whereby an increase in net lipolysis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient

In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In another aspect, the invention is a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to a cell capable of producing or metabolizing lipids under conditions permissible for metabolizing or producing lipids, and monitoring the expression of a PPARγ:RXR-regulated gene in the cell, whereby a change in gene expression of the PPARγ:RXR-regulated gene indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In another aspect, the invention is a method of screening an RTA for its capacity to affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity in a patient comprising administering the RTA to a cell containing a retinoid-regulated gene in the presence of a retinoid, and monitoring the cell for a change in the expression of the retinoid-activated gene, whereby a change in the expression of the retinoid-activated gene indicates the RTA can affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity, thereby screening the RTA for its capacity to lipodystrophy, dyslipidemia, or retinoid-associated toxicity in the patient. Such retinoid-associated toxicities include, but are not limited to, skin defects such as dry skin, alopecia (hair loss), nail defects (such as ingrown toenails), hepatotoxicity, organomegaly, hypothyroidism, leukopenia, hypercalcemia, fatiue, musculoskeletal pain, arthralgia, xerostomia, dermatitis, oral lesions and perioral lesions. Administration of the RTA to a cell "in the presence of a retinoid" does not indicate that a separate retinoid supplement must be added to the cell culture, although of course that procedure is also contemplated. Retinoid supplementation is not required because common culture media such as fetal calf serum already has a retinoid component. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In another aspect, the invention provides a method of screening a compound for potential RTA activity comprising contacting a PPARγ receptor ligand complex with the compound, and monitoring the complex for displacement of the receptor ligand from the complex, whereby a compound that displaces the receptor ligand is a potential RTA, thereby screening the compound for its potential RTA activity. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In yet another aspect, the invention provides a method of screening a compound for potential RTA activity comprising contacting a PPARγ receptor ligand complex with the compound and monitoring the complex for binding of the compound to the complex, whereby a compound that binds to the complex is a potential RTA, thereby screening the compound for its RTA activity. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In yet another embodiment, the invention provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring the mammal for an increase in serum lipids, whereby the increase in net serum lipids indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in a patient. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

The invention also provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring net fat deposition in the mammal, whereby a change in net fat deposition indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

The invention also provides a method of identifying a compound for treating RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to an RTA-sensitive mouse, and monitoring the mouse for a change in

the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to decrease lipodystrophy or dyslipidemia in the mammal and thereby treat RTA-induced lipodystrophy or dyslipidemia in a mammal. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

In another embodiment, the invention provides a method of detecting a capacity of a compound to cause RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to an RTA-sensitive mouse, monitoring the mouse for a change in expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia in the mouse, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to cause RTA-induced lipodystrophy or dyslipidemia in the mammal. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

The invention also provides a method of classifying a patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia, comprising administering RTA to the patient, monitoring the patient for a change in the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and or the activity of the gene product, an increase in fat distribution, and/or a decrease in serum lipids indicates the patient may be susceptible to RTA-induced lipodystrophy or dyslipidemia; thereby classifying the patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia. In a preferred embodiment, the RTA is a PI. In another preferred embodiment, the RTA is an NRTI.

The NRTIs which may be screened by the methods disclosed herein are dideoxy dNTP analogs that interact with HIV reverse transcriptase (RT), and compete with dNTPs during DNA synthesis. Known examples of such NRTIs include, but are not limited to, AZT, 3TC, abacavir/ABC, D4T and DDI. Of course, any NRTI may be screened by the methods of the invention.

The NNRTIs which may be screened by the methods disclosed herein are compounds that interact with the RT at an allosterically located site spatially close to the nucleoside binding site. A cooperative interaction between the two sites increases their inhibitory effect on HIV RT. Examples of NNRTIs include, but are not limited to, efavirenz (EFV), nevirapine (NVP) and delavirdine (DLV). Of course, any NNRTI may be screened by the methods of the invention.

The protease inhibitors that can be screened by the methods disclosed herein comprise any compound or composition that can inhibit a protease. Proteases are well known to one skilled in the art and comprises those proteins that have an activity comprising the capacity to hydrolyze a peptide or a peptide-like bond. For example, carboxypeptidase A is a digestive enzyme that hydrolyzes the carboxy-terminal amino peptide bond in a polypeptide. Other examples of proteases include trypsin, chymotrypsin, bromelain, chymopapain, clostripain, collagenase, elastase, ficin, kalikrein, metalloendopeptidase, papain, pepsin, peptidase, proteinase A, and proteinase K. The protease inhibitor that can be screened using the methods of the present invention can preferably be one that inhibits a protease comprising a retroviral protease. The retroviral protease can be one found in the oncovirinae subfamily of retroviruses, such as one from HTLV-I or HTLV-II (human T-cell leukemia virus type I and type II, respectively). Additionally, the protease can be one from the lentivirinae subfamily of retroviruses, such as HIV-1, HIV-II, SIV, FIV, EIAV, and CAEV (human immunodeficiency virus type I, human immunodeficiency virus type II, simian immunodeficiency virus, feline immunodeficiency virus, equine infectious anemia virus, and caprine arthritis-encephalitis virus, respectively). In one preferred embodiment of the present invention, the protease inhibitor inhibits the protease

activity of an aspartyl protease, preferably a viral aspartyl protease, and even more preferably, a HIV-I protease.

The term "inhibitors" is also familiar to one skilled in the art and is used herein to describe any compound or composition that inhibits or decreases the activity of a protease. The degree of inhibition does not have to be complete, such as completely inhibiting the activity of the protease, and therefore comprises any inhibition of the protease relative to the activity of the protease in a similar environment in the absence of the inhibitor.

Protease inhibitors are well known in the art and include, for example, amastatin, nitrobestatin, AMPSF (4-amidinophenylmethanesulfonyl fluoride), antipain, antitrypsin, aprotinin, bestatin, chymostatin, cystatin, 3,4, dichloroisocoumarin, ebelactone A, ebelactone B, elastatinal, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, EDTA, EGTA, leupeptin, α_2-microglobulin, Nle-Sta-Ala-Sta, pepstatin A, PMSF, phosphoramidon, TLCK, TPCK, soybean trypsin inhibitor, and egg trypsin inhibitor.

HIV proteases are also known in the art and include peptide analogs based on the transition state mimetic concept. (*Roberts et al.* (1990) Science 248:358-361, *Meek et al.* (1992) J. Enzyme Inhib. 6:65098, *Meek et al.* (1990) Nature (Lond.) 343:90-92, and *Mimoto et al.* (1991) 39(9):2465-2467). Additionally, some peptide derivatives incorporate an active hydroxyethylamine moiety and inhibit both HIV-I and HIV-II proteases. Some of the more well known HIV protease inhibitors includes nelfinavir, saquinavir, ritonavir, amprenavir, and indinavir.

As used herein, the term "affect" comprises an increase in lipodystrophy or symptoms or conditions associated with dyslipidemia, a decrease in lipodystrophy or symptoms or conditions associated with dyslipidemia, or any other alteration of lipodystrophy or symptoms or conditions associated with dyslipidemia. One skilled in the art will appreciate that lipodystrophy comprises peripheral fat "wasting" but can concurrently comprise fat accumulation in another region or area of a patient or subject. Therefore lipodystrophy can encompass both a decrease and an increase in fat

distribution, and can also include an unseen or undetected alteration of fat metabolism associated with the detrimental side effects of protease therapy.

The compounds or compositions that represent RTAs can be administered to a cell in any number of ways. For example, the compound or composition can be added to the medium in which the cell is growing, such as tissue culture medium for cells grown *in culture*. Alternatively, the RTA can be administered to a cell of a patient or a subject *in vivo*, or *ex vivo*. One skilled in the art will appreciate that cells will internalize the RTA in any number of mechanisms, whether the administration is to a cell *in vitro* or a cell *in vivo*, such as endocytosis or passive or active transport. Alternatively, the RTA can be specifically delivered to the interior of a cell using importation techniques such as direct injection, liposome delivery, or peptide-assisted delivery (See, e.g. U.S. 5,807,746 "Method for Importing Biologically Active Molecules into Cells", *Lin et al.*).

Alternatively, the RTA can be topically administered to a subject such as a patient or an animal. Other examples of administration of an RTA yinclude inhalation of an aerosol, subcutaneous or intramuscular injection, direct transfection of a nucleic acid sequence encoding the compound where the compound is a nucleic acid or a protein into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods include oral administration, particularly when the composition is encapsulated, or rectal administration, particularly when the composition is in suppository form. A pharmaceutically acceptable carrier includes any material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. One skilled in the art will appreciate that the mode of administration of the RTA is secondary to the core invention and is therefore not limited to any particular technique.

Any cell that can undergo adipogenesis, lipogenesis, and/or lipolysis can be used in the methods of the present invention, including, but not limited to, mesenchymal stem cells, liver cells (such as HepG2 cells), muscle cells, osteoblasts, Schwann cells, adipocytes, and preadipocytes.. Preferably, the cell is an adipocyte or a preadipocyte cell, also referred to as a preadipose cell. Primary preadipocyte cells can be isolated from the stromal vascular fraction of adipose tissue and, when treated in cell culture with a combination of adipogenic effectors, can differentiate into adipocytes (*Kirkl et al.* (1990Am. J. Physiol. 258:C206B10; for general reviews on adipogenesis see *Brun et al.* (1996Current Opinion in Cell Biology 8:826-832; *Cornelius et al.* (1994Annual Review of Nutrition 14, 99-129; *MacDougald et al.* (1995) Annual Review of Biochemistry 64:345-73; *MacDougald et al.* (1995Current Biology 5:618-21; and *Smas et al.* (1995) Biochemical Journal 309:697-710). One skilled in the art will appreciate that the cell used in the methods disclosed herein can be a mammalian primary cell or an established cell line, and more specifically a human primary cell or established cell line.

Established cell lines most frequently used as models for studies on adipocyte differentiation are generally of two types: fibroblastic multipotent cell lines that have not been committed to the adipose lineage and preadipocyte cell lines that have undergone such commitment. Established preadipocyte lines have advantages over primary preadipocytes in that they provide a homogeneous population and can be carried in culture indefinitely. These cell lines can be induced to differentiate into adipocytes that display the morphological (*Novikoff et al.* (1980 J. Cell Biol. 87:180B96) and biochemical (*Cornelius et al.* (1994Annu. Rev. Nutr. 14:99B129 and *MacDougald et al.* (1995Annu. Rev. Biochem. 64:345B73) characteristics of adipocytes *in situ*. When appropriately induced in culture, these preadipocyte cells undergo differentiation and acquire the biochemical and morphological phenotype of adipocytes (*Green et al.* (1974Cell 1:113B16; *Green et al.* (1974 Cell 3:127B33,;*Cornelius et al.* (1994Annu. Rev. Nutr. 14:99B129; and *Mandrup et al.* (1997J. Biol. Chem. 272:5367B70). Many of the same effectors that induce

differentiation of primary preadipose cells into adipocytes also induce differentiation of the established preadipocyte cell lines (*Cornelius et al.*).

Examples of committed preadipocytes that can be used in the methods disclosed herein include 3T3-L1 cells (a subclone of Swiss 3T3, *Green et al.* (1974 Cell 1:113B16), 3T3-F442A cells (a subclone of Swiss 3T3, *Green et al.* (1976Cell 7:105B13), Ob17 cells (dedifferentiated adipocytes from epididymal fat pads of C57B L/6J ob/ob mice, *Negrel et al.* (1978) Proc. Natl. Acad. Sci. USA 75:6054B58), Ob1771 cells (a subclone of Ob17, *Amri et al.* (1986) Biochem. J. 238:115B22), TA1 cells (a subclone of C3H10T1/2, *Chapman et al.* (1984) J. Biol. Chem. 259:15548B55), 30A5 cells (a subclone of C3H10T1/2, *Konieczny et al.* (1984) Cell 38:791B800), and 1246 cells (an adipogenic subclone of the CH3 mouse teratocarcinoma cell line T984, *Darmon et al.* (1981) Exp. Cell Res. 132:313B27). One skilled in the art will appreciate that other cells that have the necessary characteristics of these examples of committed preadipocytes can also be used in the methods disclosed herein. For a list of the relevant characteristics of C3H10T1/2 cells, see *Paulik et al.* ((1997) Cell Tissue Res. 290:79-87).

Examples of cells that are non-committed but with adipogenic potential that can be used with the methods disclosed herein include NIH3T3 cells (NIH Swiss mouse embryo cells, *Jainchill et al.* (1969J. Virol. 4:549B53), Swiss 3T3 cells (Swiss mouse embryo cells, *Todaro et al.* (1963) J. Cell Biol. 17:299B313), Balb/3T3 cells (Balb/c mouse embryo cells, *Aaronson et al.* (1968) J. Cell Physiol. 72:141B48), C3H10T1/2 cells (C3H mouse embryo cells, *Reznikoff et al.* (1973) Cancer Res. 33:3231B38 and *Paulik et al.* (1997) Cell Tissue Res. 290:79-87), C2C12 cells (thigh muscle of C3H mice, *Yaffe et al.* (1977) Nature 270:725B27), and G8 cells (hind limb muscle from fetal Swiss Webster mouse, *Christian et al.* (1977) Science 196:995B98). One skilled in the art will appreciate that other cells that have the necessary characteristics of these examples of non-committed cells having adipogenic potential can also be used in the methods disclosed herein.

One skilled in the art will recognize that the conditions permissible for differentiation of a preadipocyte into an adipocyte can, of course, vary depending on the specific cell type used. For example, conditions for differentiation of 3T3-L1 cells includes culturing the cells in the presence of fetal bovine serum, insulin, dexamethasone, and methylisobutylxanthine (*Student et al.* (1980J. Biol. Chem. 255:4745B50). Conditions for differentiation of 3T3-F442A and Ob17 cells includes culturing the cells in the presence of fetal bovine serum and insulin (*Spiegelman et al.* (1980) J. Biol. Chem. 255:8811B18 and *Negrel et al.*). Conditions for differentiation of Ob1771 cells includes culturing the cells in the presence of fetal bovine serum, insulin, and triiodothyronine, (*Amri et al.*). Conditions for differentiation of TA1 cells includes culturing the cells in the presence of fetal bovine serum, insulin, and dexamethasone, *Chapman et al.* (1984) and *Chapman et al.* (1985) J. Cell Biol. 101:1227B35). Conditions for differentiation of 30A5 cells includes culturing the cells in the presence of fetal bovine serum, insulin, methylisobutylxanthine, and dexamethasone, *Pape et al.* (1988) Mol. Endocrinol. 2:395B403). Conditions for differentiation of 1246 cells includes culturing the cells in the presence of insulin, methylisobutylxanthine, and dexamethasone, *Serrero et al.* (1982) Anal. Biochem. 120:351B59 and *Gao et al.* (1990) J. Biol. Chem. 265:2431B34).

For conditions permissive for the differentiation of cells that are non-committed but have adipogenic potential, see *Hwang et al.* (1997) Annu. Rev. Cell Dev. Biol. 1997. 13:231-259. In general, these lines can be committed to the adipose lineage by expression of certain transcription factors, such as members of the CAAT/enhancer-binding protein (C/EBP) and peroxisome proliferatorBactivated receptor (PPAR) families (*Freytag et al.* (1994Genes Dev. 8:1654B63 and *Lin et al.* (1994) Proc. Natl. Acad. Sci. USA 91:8757B61). The conditions for inducing C3H10T1/2 cells to undergo differentiation include administration of PPARγ ligands, RXR ligands, insulin receptor ligands, expression of adipocyte-specific proteins such as UCP, type II deiodinase, aP2, LPL, adipsin, and the beta 3 adrenergic receptor. Despite any specific differences in the differentiation conditions, there is believed to be a

similar sequence of events in the differentiation programs of all preadipocyte cell lines (*Hwang et al.*). In all cases when quiescent preadipocytes are treated with differentiation inducers, they undergo two to three rounds of cell division (clonal expansion), become growth- arrested, and then coordinately express adipocyte genes (*Cornelius et al.* (1994Annu. Rev. Nutr. 14:99B129). Monitoring for an inhibition of adipogenesis therefore not only includes monitoring phenotypic markers, but monitoring genotypic markers as well (*Butterwith, S. C.* (1994) Pharmacology and Therapeutics 61: 399-411).

Alternatively, the cell used in the methods disclosed herein can be a differentiated adipocyte. One skilled in the art will recognize that if a differentiated adipocyte is used in these methods, that cell does not have to be under preadipocyte differentiation conditions, but only be in conditions permissive for lipogenesis and/or lipolysis. Typically, lipogenesis can be stimulated by adding agents, such as insulin, insulin sensitizors, acylation stimulating protein (ASP), and other factors that increase the activity of fatty acid synthase and result in the incorporation of glycerol, fatty acids, and/or glucose into lipids (e.g., triglycerides). Lipolysis can be enhanced by adding agents that antagonize lipogenesis and/or stimulate lipoprotein lipase activity (e.g., agents that increase intracellular cyclic-AMP, such as adrenergic agonists or phosphodiesterase inhibitors). One skilled in the art will also recognize that the methods comprising monitoring lipogenesis, lipolysis, and/or PPARγ:RXR-activated gene expression are applicable to both adipocytes and preadipocytes.

Examples of the phenotypic markers associated with adipogenesis include, but are not limited to, the acquisition or accumulation of lipids from a positive or increased net lipogenesis, a negative or reduced net lipolysis, and/or increased cellular triglyceride levels. Other adipocyte markers include, but are not limited to, expression of fatty acid binding protein (aP2), expression of lipoprotein lipase (aP2), expression of adipsin, expression of PPARγ-inducible genes, expression of uncoupling protein 1, expression of type II deiodinase, expression of PEPCK, expression of leptin, and expression of glucose transporter 4 (GLUT4).

Methods of monitoring these and other phenotypic markers are well known in the art. Examples of these methods include, but are not limited to, morphological characteristics (*Novikoff et al.* (1980) J. Cell Biol. 87:180B96), and histological parameters (*Green et al.* (1979) J. Cell Phys. 101:169B72) such as staining lipids with a lipid stain such as Oil Red O (*Novikoff et al.* (1980J. Cell Biol. 87:180B96) and nile red to stain for lipid (*Kenakin et al.* (1998) Current Protocols in Pharmacology 4.6.1-4.6.36). Several biochemical parameters can also be measured to determine the extent of adipose differentiation including, but not limited to, fatty acid oxidation, glucose transport activity, glycerol-neogenesis, anaerobic or aerobic respiration, membrane potential changes, thermogenesis, lipolysis, and lipogenesis (*Lenhard et al.* (1997) Biochemical Pharmacology 54, 801-808).

Examples of the genotypic markers associated with adipogenesis include, but are not limited to, expression of stearoyl-CoA desaturase gene 1 (*Casimir et al.* (1996) J. Biol. Chem. Nov 22; 271 (47):29847), expression of the phosphoenolpyruvate carboxykinase gene (*Tontonoz et al.* (1995Mol. Cell. Biol. 15:351B57), expression of the aP2 gene (an adipocyte-specific fatty acid binding protein, *Tontonoz et al.* (1994Genes Dev. 8:1224B34), lipoprotein lipase (*Schoonjans et al.* (1996 Biochim. Biophys. Acta 1302:93B109), alkaline phosphatase, and other PPAR:RXR (peroxisome proliferator activated receptor:retiniod X receptor) activated genes. In a preferred embodiment of the present invention, the PPAR receptor is a PPARγ receptor.

Methods of monitoring these and other genotypic markers are well known in the art. Examples of these methods include, but are not limited to, monitoring expression of a gene, such as the transcription of the gene, the translation of the RNA transcribed from the gene, or both. (*Mandrup et al.* (1997) Proc. Natl. Acad. Sci. USA 93:4300B5). These parameters can be monitored for their relative rates, or as a function of the amount of the product of the particular reaction or process. The expression of the gene, and therefore genotypic markers, can also be monitored by less direct methods as well. For example, the stability of an RNA or a protein can be monitored, or the ability of the RNA to be transported from the nucleus to the

cytoplasm can be monitored. The post- transcriptional and/or post-translational processing of an RNA and/or a protein can also be monitored. Other parameters that can be monitored include binding of a ligand to it's receptor, preferably a nuclear receptor such as PPARγ, RAR, and/or RXR. Alternatively, the displacement of a ligand from it's receptor can be monitored. The activity or levels of a protein or polypeptide encoded by a nucleic acid that can be regulated, affected by, or associated with adipogenesis can also be monitored.

PPARs are a class of nuclear hormone receptors that control transcription of several genes involved in lipid and carbohydrate metabolism. Members of the PPAR family bind as heterodimeric complexes with the retinoid X receptors (RXRs) to PPAR response elements (*Mangelsdorf et al.* (1995) Cell 83:841B50). Such heterodimeric complexes are denoted herein as PPARγ:RXR.

Transcriptional activation by PPARs is strongly induced by the binding of a ligand to the receptor, and the transactivation potential of all the PPARs is stimulated synergistically by the presence of both insulin and a PPAR ligand (*Shalev et al.* (1996) Endocrinology 137:4499B502 and *Zhang et al.* (1996) J. Biol. Chem. 271:31771B74). Examples of PPAR ligands includes, but is not limited to a thiazolidinedione (*Teboul et al.* (1995 J. Biol. Chem. 270:28183B87). In a specific embodiment, the thiazolidinedione is BRL49653 (*Lehmann et al.* (1995) J Biol Chem 270:12953-6). Another example of a thiazolidinedione is troglitazone (*Lenhard et al.*, (1997) Biochem Pharmacol 54:801-8). Other PPAR ligands include prostanoids such as prostaglandin J2 (*Kliewer et al.* (1995) Cell 83:813-9), non-steroidal anti-inflammatory drugs such as indomethacin (*Lehmann et al.* (1997 J Biol Chem 272:3406-10), fatty acids and eicosanoids (*Kliewer et al.* (1997) Proc Natl Acad Sci U S A 94:4318-23), and N-(2-Benzoylphenyl)-L-tyrosine PPARγ agonists (*Henke et al.* (1998) J Med Chem 41:5020-3).

The effects of retinoids are mediated by retinoid receptors. There are at least two specific classes of retinoid nuclear hormone receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). RARs can bind to DNA as heterodimers

with RXRs. PPARs can also heterodimerize with RXR. Therefore the effect of retinoid receptors and PPAR receptors can be affected by retinoid receptor ligands, such as RAR and RXR ligands. Retinoic acid receptor (RAR) agonists inhibit adipogenesis (*Salazar-Olivo et al.* (1994) Biochem Biophys Res Commun 204, 257-63) whereas retinoid X receptors (RXR) induce adipogenesis (*Schulman I.G.* (1998) " Mol Cell Biol 18:3483-94). General examples of ligands that can bind retinoid receptors includes, but is not limited to, rexinoids, such as LGD1069 and cis-9-retinoic acid, and CH55. Examples of ligands that bind to RXRs include, but are not limited to, retinoids, such as LGD1069 and cis-9-retinoic acid. Examples of ligands that bind to RARs include, but are not limited to all-trans retinoic acid, cis-9-retinoic acid, Am80, Am580, andCH55. In a preferred embodiment of the present invention, a phenotypic marker associated with RAR:RXR activation that is monitored is the activity of alkaline phosphatase or any other retinoid regulated gene.

Transcriptional activation assays useful for the screening of RTAs for their capacity to affect lipodystrophy or dyslipidemia include transfecting constructs into cells which comprise a peroxisome proliferator response element (PPRE) conjugated to a sequence encoding a reporter protein. For a general review on PPRE's, see *Green et al.* (1994) Mol Cell Endocrinol 100:149-53). For example, a PPRE activated by a PPAR:RXR heterodimer can be conjugated to a nucleic acid encoding alkaline phosphatase. The ligand which activates the PPAR:RXR heterodimer can be added in the presence and absence of an RTA to determine the effect of the RTAon transcriptional activation by measuring alkaline phosphatase activity. In a related embodiment, a cotransfection assay could be utilized (See U.S. Patent Nos. 4,981,784 and 5,071,773) whereby one plasmid encodes the hormone receptor and a second plasmid encodes the PPRE conjugated to the reporter protein.

One can also screen potential RTAs by their capacity to displace PPARγ receptor ligands from the receptor/ligand complex, whereby a compound that displaces a ligand from the complex is a potential RTA. For example, the receptor ligand can be radiolabeled and complexed with the receptor and the compound then added to that

mixture. If the potential RTA displaces the ligand, free ligand can be measured and correlated to the concentration of the potential RTA required for that particular percent of the ligand displaced. One skilled in the art will recognize there are multiple variations on this general experimental approach that can be used to determine whether a potential RTA has the capacity to displace the ligand from the receptor/ligand complex.

Similarly, one skilled in the art will recognize that assays comprising binding inhibition or competitive binding can be used to screen potential RTAs. For example, one can label a potential RTA, contact a receptor with the labeled potential RTA such that the RTA and the receptor form a complex, and then add increasing amounts of a receptor ligand to the complex and determine whether the potential RTA inhibits binding of the ligand to the receptor. Alternatively, the potential RTA and the ligand can be mixed with the ligand in varying concentrations to determine whether the potential RTA inhibits binding of the ligand to the receptor. One skilled in the art will also appreciate that there are many variations of binding assays and competitive binding assays that can be used in the methods disclosed herein. An example of one such method is described below in the Example whereby Saquinavir inhibits binding of BRL49653 to PPARγ.

The invention also provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a mammal, comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring the mammal for an increase in serum lipids, whereby the increase in net serum lipids indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in the mammal. Preferably, the RTA is a protease inhibitor. In one embodiment, the mammal is maintained under high-fat diet conditions. One of skill in the art will readily understand how to determine whether the mammal has undergone a change in serum lipids. For example, a change in serum lipids may be indicated by a change in serum triglycerides, free fatty acids, glycerol, or cholesterol.

In one embodiment, the mammal used in the above-described screening assay is

a mouse. Preferably, the mouse will have the obesity-related characteristics of a AKR/J mouse.

The invention also provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a mammal, comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring net fat deposition in the mammal, whereby a change in net fat deposition indicates RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the mammal. Preferably, the RTA is a protease inhibitor. In one embodiment, the mammal is maintained under high-fat diet conditions. One of skill in the art will readily understand how to determine whether the mammal has undergone a change in net fat deposition. Such a change may, for example, be indicated by a change in the weight of fat pads. In one embodiment, the fat depots are interscapular or epididymal fat depots. A change in net fat deposition may also be indicated by a change in expression or activity of proteins produced by adipocytes.

In one embodiment, the mammal used in the above-described screening assay is a mouse. Preferably, the mouse will have the obesity-related characteristics of a AKR/J mouse.

The invention also provides a method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a mammal, comprising administering the RTA to a mammal susceptible to diet-induced obesity, and monitoring the mammal for an increase in blood urea nitrogen or glucose, whereby the increase in blood urea nitrogen or glucose indicates the RTA has the capacity to affect lipodystrophy or dyslipidemia in the mammal. In a preferred embodiment, the mammal is maintained under high-fat diet conditions. Preferably, the RTA is a protease inhibitor.

In one embodiment, the mammal used in the above-described screening assay is a mouse. Preferably, the mouse will have the obesity-related characteristics of a AKR/J mouse.

In yet another embodiment, the invention provides a method of an RTA for its

capacity to affect lipodystrophy or dyslipidemia in a mammal, comprising administering the RTA to a mammal susceptible to diet-induced obesity, wherein the mammal contains a retinoid-activated gene under activation conditions, and monitoring the mammal for a change in the expression of a retinoid-activated gene, whereby a change in the expression of the retinoid-activated gene indicates RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the mammal . In one embodiment, the mammal is maintained under high-fat diet conditions. In a preferred embodiment, the RTA is a protease inhibitor.

In a preferred embodiment, the retinoid-activated gene is a gene which encodes alkaline phosphatase. In another prefered ambodiment, the retinoid-activated gene is activated by a retinoid nuclear receptor.

In one embodiment, the mammal used in the above-described screening assay is a mouse. Preferably, the mouse will have the obesity-related characteristics of an AKR/J mouse.

The present invention further contemplates a transgenic animal whose somatic cells comprise and express a transgene conferring sensitivity to an RTA, wherein the total native and transgene expressed in the transgenic animal is higher than the native gene expressed in a non-transgenic animal, which transgenic animal has a phenotype of increased sensitivity to the RTA. In a preferred embodiment, the RTA is a protease inhibitor.

The invention also provides a transgenic animal whose somatic cells comprise and overexpress ubiquitously in all tissues a transgene conferring sensitivity to an RTA, wherein the total native and transgene expressed in the transgenic animal is higher than the native gene expressed in a non-transgenic animal, which transgenic animal has a phenotype of increased sensitivity to the protease inhibitor. Preferably, the RTA is a protease inhibitor.

In the transgenic animals of the invention, the transgene may be a retinoid-activated gene. In a preferred embodiment, the gene is activated by a retinoid nuclear

receptor.

In another embodiment, the transgene is a PPARγ:RXR-activated gene. In yet another embodiment, the transgene is a protease inhibitor regulated gene.

As used herein, "transgenic animal" describes a non-human animal which has been altered to express a transgene conferring sensitivity to a protease inhibitor, wherein the transgenic animal has an increased sensitivity to the protease inhibitor. In a preferred embodiment, the transgenic animal of the present invention is a mouse. Such transgenic animals can be produced according to methods well known in the art and as described herein for introducing exogenous DNA into the germ line of an animal or "knocking out" a functional gene product. The transgenic animals of this invention can be used in the screening methods described herein to identify compounds which may be used to treat lipodystrophy or dyslipidemia in a patient. The transgenic animals of this invention can also be used in detecting a capacity of a compound to express protease inhibitor-induced lipodystrophy or dyslipidemia in a patient.

Thus, the invention provides a method of identifying a compound for treating RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to an RTA y-sensitive mammal, and monitoring the mouse for a change in the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to decrease lipodystrophy or dyslipidemia in the mammal and thereby treat RTA-induced lipodystrophy or dyslipidemia in a mammal. In a preferred embodiment, the RTA is a protease inhibitor.

The invention also provides a method of detecting a capacity of a compound to express RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to a protease inhibitor-sensitive mammal, monitoring the mammal for a change in expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia in the mammal a change in fat

distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to express RTA-induced lipodystrophy or dyslipidemia in the patient. In a preferred embodiment, the RTA is a protease inhibitor.

In yet another embodiment, the invention provides a method of classifying a patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia, comprising administering the protease inhibitor to the patient, monitoring the patient for a change in the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and or the activity of the gene product, an increase in fat distribution, and/or a decrease in serum lipids indicates the patient may be susceptible to protease inhibitor-induced lipodystrophy or dyslipidemia; thereby classifying the patient as being susceptible to protease inhibitor-induced lipodystrophy or dyslipidemia. In a preferred embodiment, the RTA is a protease inhibitor.

The following Examples are set forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the methods claimed herein performed, and is intended to be purely exemplary of the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C and pressure is at or near atmospheric.

EXAMPLES

Example 1: HIV Protease Inhibitors Block Adipogenesis and Increase Lipolysis

HIV Protease inhibitors inhibit lipid accumulation in C3H10T1/2 cells

Lipid accumulation during adipocyte differentiation involves the coordinated action of triglyceride synthesis (lipogenesis) and hydrolysis (lipolysis). Thus, it is necessary to determine which metabolic pathway protease inhibitor-treatment affected. To determine the effect of the protease inhibitors (PIs) on lipolysis, C3H10T1/2 mesenchymal stem cells were grown in Dulbecco's modified Eagle medium-high glucose containing 10% fetal calf serum. The cells were cultured in 96-well microtiter plates (12×10^3 cells/cm^2) and treated with 1 μM BRL49653, 1 μM LGD1069, and 1 μM insulin (conditions which suppress lipolysis). At the same time, vehicle (dimethylsulfoxide, DMSO) or test compounds were suspended to 40 mM in DMSO and serially diluted from 40 μM to < 40 nM into the culture medium of the preadipocyte stem cells. The cells were cultured for 7 days and lipolysis was measured as described in *Lenhard et al.* (Biochem. Pharmacol. 54:801-808 (1997). As shown in Figure 1, nelfinavir, nitonavir, and saquinavir stimulated lipolysis in a concentration dependent manner, whereas amprenaivr and indinavir had no effect on lipolysis. Thus, this assay can be used to screen, identify, and rank PIs according to their capacity to alter fat catabolism (lipolysis).

Although PIs stimulate lipolysis, it is unknown whether the PIs stimulate lipolysis in the differentiating preadipocytes or whether the effect requires protein synthesis. Thus, adipocytes were prepared from C3H10T1/2 clone 8 murine fibroblasts (ATCC) as described in *Paulik et al.* (1997) Cell Tissue Res. 290:79-87. Isoproterenol, an andrenergic agonist that stimulates lipolysis, was used as a positive control. Saquinavir, nelfinavir, and isoproterenol stimulated lipolysis in mature adipocytes. (Figure 2). Cyclohexamide had no effect, indicating protein synthesis was not required for stimulation of lipolysis in adipocytes by PIs. Not all PIs stimulated lipolysis, demonstrating this assay can be used to screen, identify, and rank PIs according to their capacity to alter fat catabolism.

These data clearly differ from that of *Carr et. al.*, since that reference states PIs alter metabolism by inhibiting synthesis of 9-cis retinoic acid and thus, the lack of an

agonist causes RXR to remain inactive. In contrast, the data discussed above demonstrate that several PIs inhibit adipogenesis in the presence of RXR agonists. Moreover, unlike the hypothesis of *Carr et al.*, the above data demonstrate that the ability of PIs to stimulate lipolysis is independent of protein synthesis and therefore *Carr et al.*'s hypothesis may not be valid. Also in contrast to the above results, *Gagnon et al.*, suggest ritonavir and indinavir stimulate adipogenesis as much as 10-40% in 3T3-L1 cells. There are several differences between 3T3-L1 and C3H10T1/2 cells which may explain this discrepancy. 3T3-L1 cells require insulin, glucocorticoids, and a cAMP elevating agent (e.g., isobutylmethylxanthine) to differentiate whereas C3H10T1/2 cells require insulin and agonists for the RXR/PPARγ heterodimer to differentiate into adipocytes. Moreover, 3T3-L1 cells express a phenotype similar to white adipose tissue whereas C3H10T1/2 cells express a phenotype similar to brown adipose tissue. Thus, the effects of PIs on fat metabolism may vary between fat depots. Consistent with this hypothesis, PI therapy is associated with a loss of fat from the face and limbs but an increase in fat in the back of the neck and abdomen. These results point to important differences between various fat depots in the development of PI-associated lipodystrophy.

Total triglyceride assays and results using differentiating preadipocytes

Since some PIs stimulate lipolysis, it is possible that PI- treatment could increase total triglyceride accumulation *in vitro*. To test this hypothesis, one day after passage of C3H10T1/2 cells into 96-well microtiter plates (12.5 x 10^3 cells/cm^2), the cells were treated with 1 μM BRL49653, 1 μM LGD1069, and 1 μM insulin (conditions which stimulate triglyceride accumulation). At the same time, test compounds were suspended to 40 nM in DMSO and serially diluted from 40 μM to < 40 nM into the culture medium of the preadipocyte stem cells. The cells were cultured for 7 days and the accumulation of triglycerides was measured as described in *Lenhard et al.* (Biochem. Pharmacol. 54:801-808 (1997). As shown in Figure 3, nelfinavir, nitonavir, and saquinavir inhibited triglyceride accumulation in these cells. The PIs inhibited triglyceride accumulation to varying degrees. Thus, this assay can be used to

screen, identify, and rank PIs according to their capacity to alter total triglyceride, and therefore fat synthesis.

Total glyceride assays and results using adipocytes

Since the inhibition of triglyceride accumulation by PI administration may result from stimulation of lipolysis and/or inhibition of lipogenesis, it was therefore necessary to determine whether the PIs have an effect on lipogenesis as well as the effects on lipolysis. Adipocytes were therefore prepared as described in *Paulik et al.* (1997) Cell Tissue Res. 290:79-87. Mature adipocytes were treated for three days with the indicated compounds (20 μM) and lipogenesis was measured as described in *Lenhard et al.* As shown in Figure 4, nelfinavir, nitonavir, and saquinavir inhibited lipogenesis to varying degrees. Thus, this assay can be used to screen, identify, and rank PIs according to their capacity to alter lipogenesis, and therefore fat synthesis.

HIV protease inhibitors block expression of adipose-specific genes in C3Hl0T1/2 cells

Lipoprotein lipase (LPL) contains a PPARγ/RXR recognition site in its promotor and is one of the earliest genes induced during adipogenesis. Similarly, the expression of aP2, an adipocyte specific fatty acid binding protein, is directly regulated by activation of the PPARγ/RXR heterodimer. To characterize the effects of HIV protease inhibitors on PPARγ activity at the molecular level, Northern blot analysis of LPL and aP2 was performed. One day after passage into 96-well microtiter plates (12.5 x 10^3 cells/cm^2), C3H10T1/2 cells were treated with 1 μM BRL49653, 1 μM LGD1069, and 1 μM insulin (conditions which activate endogenous PPARγ). At the same time, cells were treated with 20 μM of test compounds or vehicle (dimethylsulfoxide, DMSO). The cells were cultured for 7 days and total RNA was isolated using using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Ten μg of RNA was electrophoresed in agarose gels and transferred to nitrocellulose. The blot was probed with mouse aP2 and lipoprotein lipase (LPL) probes labeled via the random-priming technique (Prime-It II Kit, Stratagene, La Jolla, CA) with [α^{32}P]dCTP. The autoradigraphs were analysed with a Bicrad Imaging System. As shown in Figure

5, fat-specific mRNAs encoding LPL and aP2 were greatly reduced in cells treated with nelfinavir, ritonavir, and saquinavir whereas amprenavir and indinavir had no effect. Thus, this assay can be used to screen, identify, and rank PIs according to their capacity to alter expression of genes regulating fat metabolism and, in particular, those genes regulated by PPARγ.

HIV proteases increase the activity of alkaline phosphatase in C3Hl0T1/2 cells.

We tested the effects of PIs on a retinoic acid responsive gene, alkaline phosphatase (ALP), by culturing C3H10T1/2 preadipocytes for seven days in the presence of various PIs (amprenavir, nelfinavir, ritonavir, saquinavir, or indinavir) and retinoids (cis 9-retinoic acid, ATRA, AGN 193109, or CH55). Subsequently, ALP activity was measured as described in *Paulik et al.* Whereas ATRA (EC50 = 650 nM) and cis 9-retinoic acid (EC50 = 3.4 μM) stimulated ALP activity, the PIs had no effect on ALP in the absence of retinoids. Amprenavir (≤20 μM), in combination with cis 9-retinoic acid or ATRA, also had no effect on ALP (Figure 6). However, nelfinavir, saquinavir, and ritonavir inhibited ALP (IC_{50}s = 8-17 M) when combined with 1 μM cis 9-retinoic acid (Figure 6). Since cis 9-retinoic acid was added exogenously, the inhibitory effect of PIs can not be due to inhibition of cis 9-retinoic acid synthesis. This data reinforces the previous observation that *Carr et al.* did not disclose, teach, or suggest methods for screening PIs.

Indinavir's effect on ALP was remarkably different than the other PIs. Indinavir (EC50 = 8 ± 3 μM) increased ALP in combination with 100 nM ATRA, but not cis 9-retinoic acid (Figure 7). This effect was blocked by the RAR-antagonist, AGN 193109, indicating indinavir stimulated RAR-signaling. Unlike ATRA, the RAR-selective agonist, CH55, does not bind to CRABP. Indinavir (20 μM) did not potentiate ALP activity in combination with CH55 (Figure 8). These results support the observation that indinavir displaces ATRA from CRABP. We propose that the ATRA displaced from CRABP becomes available to activate RAR-responsive genes. As shown by others, increased RAR-activity induces hypertriglyceridemia.

Our data demonstrate the effects of PIs on retinoid-signaling are not class-specific. Nelfinavir, ritonavir, and saquinavir inhibited, indinavir stimulated, and amprenavir had no effect on ALP activity.

Binding assay for screening potential protease inhibitors.

Test compounds were assayed for competitive-inhibition of BRL49653 binding to human PPARγ ligand-binding domain as described in Nichols et al. (*Anal. Biochem. 257:* 112-119 (1998)). Ligand-binding to the human RXR ligand-binding domain was measured using a scintillation proximity assay similar to that described for PPARγ (Nichols et al., *Anal. Biochem. 257:* 112-119 (1998)). Briefly, biotinylated RXRα was immobilized on streptavidin-modified scintillation proximity assay beads followed by incubation with 2.5 nM 9-cis [^{3}H]-retinoic acid and various concentrations of PIs in 96-well polypropylene plates. The plates were incubated for 1 hour at room temperature and bound radioactivity was determined in a Wallac 1450 Microbeta counter. The data was analyzed as previously described (Nichols et al., *Anal. Biochem. 257:* 112-119 (1998))

Saquinavir had an IC50 = 12.7 ± 3.2 μM in this binding assay (Figure 9). In contrast, amprenavir had no effect in this binding assay. Thus, PIs can be screened, characterized, and ranked according to their ability to bind PPARγ for the purpose of identifying protease drug candidates.

Example 2: Influence of Diet on Metabolic Abnormalities in Mice Treated with HIV Protease Inhibitors.

The PIs used in these studies were obtained from the Medicinal Chemistry Department at GlaxoWellcome Inc. (Research Triangle Park, North Carolina).

Experimental animal protocol:

Age and weight matched male AKR/J mice (Jackson Labs, Bar Harbor, ME) were housed 5 animals/cage at 72° F and 50% relative humidity with a 12 hr light and dark cycle. Starting at 4 weeks of age the animals (8 animals/group) were fed low fat diet (11.6% kilocalories as fat; NIH R&M/Auto 6F-Ovals 5K07, PMI Feeds^7 Inc.,

Richmond, Indiana) or high-fat diet (58% kilocalories as fat; D12331, Research Diets, Inc., New Brunswick, NJ). Three-week continuous release pellets containing 20 mgs of PIs were prepared by Innovative Research of America (Sarasota, Fla). Trochar was used to implant three pellets subcutaneously in the back of each mouse starting at 8 weeks of age according to the manufacturer's specifications (Innovative Research of America, Sarasota, Fla). After 2 weeks the animals were anesthetized with isofluorane, blood drawn by cardiac puncture, and non-fasting measurements of glucose, total cholesterol, triglycerides, non-esterified free fatty acids (NEFAs), glycerol, alkaline phosphatase, bilirubin, blood urea nitrogen (BUN), pancreatic lipase, β-hydroxybutyric acid, insulin, C-peptide and leptin were obtained. All blood chemistry tests, with the exception of insulin, C-peptide and leptin, were determined as previously described (*Lenhard et aL.* "The RXR agonists L0100268 causes hepatomegaly, improves glycaemic control and decreases cardiovascular risk and cachexia in diabetic mice suffering from pancreatic beta-cell dysfunction" Diabetolgia 1999; 42: W-554). Serum insulin, C-peptide and leptin measurements were obtained from Linco Research, Inc. (St. Charles, MO). Organ weights were determined at the end of the study. Serum concentrations of the PIs were determined by operating a mass spectrometer in the LC/MS/MS mode. The data were calculated as the mean and standard error (SEM) from experiments performed on eight animals per treatment group. Two-tailed tests were performed to calculate P values. Correlation coefficients were determined by regression analysis using Excel on a personal computer. This research complies with the principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and company policy on the care and use of animals and related codes of practice.

Insulin and glucose responses

Serum glucose and insulin concentrations and the insulin resistance index increased upon feeding a high-fat diet (Table 1). As shown in Table 1, the effects of PIs on glucose, insulin and the insulin resistance index were different in the high and low fat fed animals. Although the various PIs had no significant effect on glucose and insulin concentrations in high fat fed animals, indinavir (IDV) and nelfinavir (NLV)

increased serum glucose in low fat fed animals. Moreover, saquinavir (SQV), IDV and NLV decreased serum insulin and the insulin resistance index in low fat-fed animals. The difference in glucose and insulin concentrations among the amprenavir (APV) and placebo treated groups was not statistically significant (P>0.05, Table 1). Regression analysis of the effects the PIs had on glucose, insulin and the insulin resistance index revealed no significant correlation between the low and high fat fed mice (P>0.2). C-peptide and leptin remained unchanged among the treatment groups.

Lipid responses

Increased intake of dietary fat caused an increase in serum concentrations of free fatty acids, glycerol, cholesterol and pancreatic lipase but not triglycerldes in AKR/J mice (Table 2). Moreover, IDV and NLV treatment significantly increased free fatty acids, glycerol and lipase in both high and low fat fed animals, whereas APV and SQV had less effect (Table 2). With diet as the dependent variable, the correlation coefficient for NEFAs was 0.92 (P=0.02), indicating that PIs have the same effect on NEFAs in high and low fat fed animals. In contrast, the effects of the PIs on triglycerides were different in the high and low fed animals (Table 2). In high fat fed animals, treatment with SQV, IDV and NLV increased serum triglycerides, whereas APV had no effect. However, triglycerides decreased or remained unchanged upon PI treatment of low fat fed animals. Similarly, cholesterol increased in SQV and IDV treated high fat fed animals and remained unchanged in low fat fed mice (Table 2). When high and low fat fed animals were compared, the correlation coefficient for triglycerides (r=0.1, P=0.8) and cholesterol (r=0.6, P=0.3) were not statistically significant. Finally, PI treatment of animals on either diet had little effect on serum concentrations of -hydroxybutyric acid, a product generated by fatty acid oxidation.

Clinical Toxicology

The effects on changing diet on the relative safety of PIs was performed using biochemical tests for liver (alanine aminotransferase, ALT; alkaline phosphatase, ALP; bilirubin), heart/muscle (lactate dehydrogenase, LDH), and kidney (blood urea nitrogen, BUN) (Table 3). In both low and high fat fed mice ALP, bilirubin, LDH and

ALT were greater in the IDV and NLV than the APV and SQV treated animals. Similarly, IDV and NLV were more effective than APV and SQV at increasing BUN in fat-fed animals. In contrast, BUN decreased in low fed animals treated with IDV and NLV. BUN was negatively correlated with ALP in the PI treated low fed mice($r = -0.88$, $P=0.05$) and positively correlated with ALP in the PI treated high fat fed mice ($r = 0.87$, $P=0.05$). These analyses demonstrate the opposite effects that PIs have on ALP and BUN in animals fed high and low fat diets.

Fat depot weights:

A high-fat diet increased interscapular and epididymal fat mass by 75 and 425 % compared to low-fat diets. Analysis of low fat fed animals revealed SQV and NLV treatment increased interscapular brown fat mass by 28% ($P=0.04$) and 32% ($P = 0.02$), respectively, relative to control animals (Table 4). Similarly, SQV and IDV treatment of low fat fed animals increased epididymal white fat mass by 40% ($P = 0.02$) and 48% ($P=0.04$), respectively (Table 4). The effects of APV and IDV on interscapular fat mass and APV and NLV on epididymal fat mass were not significant in low fat fed mice ($P > 0.05$). Similarly, none of the PIs had a significant effect on epididymal fat mass in high fat fed mice ($P \geq 0.1$). With diet as the dependent variable, the correlation coefficient for epididymal fat mass in low and high fat fed mice was 0.07 ($p=0.9$). In contrast, the correlation coefficient for interscapular fat mass was -0.89 ($p = 0.04$), indicating interscapular fat mass was negatively correlated in PI treated low and high fat fed mice.

Plasma concentrations of PIs

The plasma concentrations of the PIs were measured at necropsy to determine if diet altered the exposure levels of the animals to each drug. As shown in Table 5, diet had no significant effect on the plasma concentrations of APV, IDV or NLV. In contrast, there was 2.5 fold more SQV in plasma of animals fed low fat diet compared to high fat diet. Thus, high fat diet decreased the plasma concentrations of SQV, but not the otherPIs, in mice. Moreover, high and low fat fed animals had significantly less exposure to NLV than the other PIs.

Discussion

Feeding AIKRIJ mice low (11.6% kilocalories as fat) and high (58% kilocalories as fat) fat diets produced marked differences in the effects of protease inhibitors on metabolism. For example, SQV, IDV and NLV treatment decreased insulin levels and APV and SQV treatment lowered serum triglyceride concentrations In low but not high fat fed animals. Moreover, serum BUN levels, as well as interscapular fat mass, were negatively correlated in PI treated low and high fat fed mice. These observations indicate diet is a confounding factor affecting several side effects caused by PI treatment of mice. However, several adverse reactions brought about by PI treatment were unaffected by changing diet. In particular, IDV and NLV treatment increased NEFAs, glycerol, lipase, ALP or bilirubin in both low and high fat fed mice. These results are consistent with the observation that IDV therapy elevates NEFAs and bilirubin in AIDS patients. Further, these observations indicate that the adverse events associated with PI treatment may belong to two categories, one that is influenced by changes in diet and another that is independent of diet.

It is possible that decreased serum insulin levels lead to increased serum glucose levels in IDV and NLV treated low fat, but not high fat fed mice. Similarly, as serum insulin levels were less and NEFA and glycerol levels were greater in the PI treated low fat fed animals. It is possible these changes result from a decrease in the antilipolytic effects of insulin on adipose tissue. However, this hypothesis is inconsistent with the observations that 1) adipose tissue weight increased in low fat fed animals with IDV and NLV and 2) insulin levels remained unchanged while NEFAs increased in high fat fed mice treated with IDV and NLV. Similarly, the possibility that these agents elevate NEFAs by inhibiting fatty acid β-oxidation does not support the observation that β-hydroxybutyric acid concentrations remained unchanged after treatment with PIs. An explanation consistent with the data is that increased pancreatic lipase, an enzyme that hydrolyzes diglycerides, may cause serum NEFAs and glycerol to increase in NLV and IDV treated animals. Since serum triglycerides and NEFAs increased in high fat fed animals, it is possible that alternate pathways (e.g., increased VLDL secretion) contribute to the metabolic changes observed with high fat diet.

A comparison between the PIs revealed several differences in their *in vivo* properties. For example, the serum concentration of SQV, but not the other PIs, significantly decreased with a high fat diet relative to a low fat diet. Thus, diet may influence the pharmacokinetic properties of SQV more than the other PIs. Similarly, differences in relative potency may influence differences between the PIs. For instance, there was more APV and SQV than IDV and NLV in the serum from mice fed a high fat diet. However, IDV and NLV increased serum triglycerides and fatty acids more than APV and SQV in mice fed a high fat diet. Thus, high fat fed mice were more susceptible to developing dyslipidemia when treated with IDV or NLV than APV or SOV. Since the effects on mice varied among the different PIs used in this study, the various PIs should be considered as distinct agents with unique pharmacological profiles.

Although PI treatment may lead to an increase in abdominal and dorsocervical fat in some HIV patients, changes in fat deposition have not been reported previously in PI-treated rodents (*Miller et al.* "Visceral abdominal-Ut accumulation associated with use of indinavir. Lancet 1998; 351: 871-875 and *Lo et al.* "'Buffalo Hump' in men with HIV-1 infection" Lancet 1998; 351: 867-870). The observation that IDV, SQV, and NFV increased either interscapular or epididymal fat mass in only low fat fed mice raises the possibility that susceptibility to PI-Induced fat redistribution is, in part, determined by diet. It is also possible that genetic and other environmental factors (e.g., temperature or stress) affect fat redistribution, Future studies comparing the effects of PIs in various inbred strains of rodents (e.g., obesity resistant SWR/J and obesity prone AKR/J mice) should provide insight into the influence that genetics has on PI-induced fat redistribution.

The observation that IDV increased BUN in high fat fed mice indicates these animals may be prone to IDV-associated nephrotoxicity. These results are consistent with several reports showing indinavir therapy causes crystallization and stone formation in the urinary tract and renal insufficiency in the clinic (*Tashima et al.* "Indinavir nephropathy" N Engl J Mod 1997;336:138-40, *Kopp et al.* "Crystalluria and

urinary tract abnormalities associated with indinavir" Ann Intern *Med* 1997;127:119-125, and *Grabe et al.* "Indinavir-induced nephropathy" Clin Nephrot 1999; 51:181-3, *Hanabuse et al.* "Renal atrophy associated with long-term treatment with indinavir" N. Engl. J Med 1999,340:392-3). The finding that BUN decreased in IDV treated low fat fed mice indicates that modification of diet may alter the susceptibility to kidney problems. Indeed, diet and nutrition are important in the management of individuals with renal disease (Drukker A. "The progression of chronic renal disease: immunological, nutritional and intrinsic renal mechanisms" J Mad Sci 1997; 33:739-43). This raises the possibility that IDV-associated nephrotoxicity may be alleviated by modification of the diet. Currently, adequate hydration is recommended to prevent nephrolithiasis in patients treated with indinavir (Indinavir Sulftte. In: Physicians *Desk Reference, Edition* 53. Edited by *Sifton et al.* Montvale: Medical Economics; 1999: 1762-66).

These data indicate that the various PIs have different effects in mice fed high and low fat diets. As diet alters susceptibility to dyslipidemia in humans and the side effects of PIs in mice, changes in diet may decrease the risk of developing adverse reactions to PIs in humans.

Example 3: Isolation of genes involved in genetic predisposition towards lipodystrophy:

AKR/J mice show distinct differences in fat metabolism upon treatment with PIs than SWR/J mice. AKR/J and SWR/J mice can therefore be interbred to produce an F_2 population, and male mice can be selected for subsequent phenotypic and genotypic evaluation. (*York et al.*, "Inherited non-autosomal effects on body fat in F2 mice derived from an AKR/J X SWR/J cross" Mammalian Genome 8:726-730 (1997), *West et al.*, "Genetics of dietary obesity in AKR/J X SWR/J mice: segregation of the trait and identification of a linked locus on chromosome 4" Mammalian Genome 5:546-552 (1994), and *West et al.*, "Dietary obesity linked to genetic loci on chromosomes 9 and 15 in a polygenic mouse model" J. Clin. Invest. 94:1410-1416 (1994)). Pups can

be weaned and placed in individual cages. The mice can be given ad libitum access to water and food, such as Purina Rodent Chow (#5001, Ralston Purina, St. Louis, Missouri), until the age of five weeks, then switched to either a low-fat diet, such as 11.6% kilocalories as fat (NIH R&M/Auto 6F-Ovals 5K07, PMI Feeds[7] Inc., Richmond, Indiana) or a high-fat diet, such as 58% kilocalories as fat (D12331, Research Diets, Inc., New Brunswick, NJ).

Mice can then be treated with PIs, such as by implantation of three-week continuous release pellets, starting at approximately 8 weeks of age. After 2 weeks of treatment, blood samples can be obtained from the animals for measuring serum lipids and lipase, as previously described. At approximately 12 weeks of age, the animals can be sacrificed dissected to remove and weigh the epidydimal and intrascapular fat pads. A frequency distribution can be determined for fat pad weight for both parental and F_2 mice, and similar frequency distributions for levels of serum lipids and lipase can be determined. Genotyping and QTL (Quantitative Trait Loci) analysis can be done on the 10% tails of the phenotype distribution. (*West et al.*, 1994).

DNA can be extracted from spleens of animals by standard phenol/chloroform extraction methods. Genotyping can be done, for example, by amplification of genomic DNA with SSLP (Simple Sequence Length Polymorphism) primers using established PCR (Polymerase Chain Reaction) conditions. For example, the SSLP primers used can be the 63 that have previously been shown to display polymorphisms between the parental AKR/J and SWR/J strains. (*West et al.*, 1994). Lengths of SSLP amplification products can be determined by gel electrophoresis. One skilled in the art can analyze this data using the Mapmaker/QTL program in order to perform a multipoint interval analysis across linkage groups and calculate the likelihood of odds (LOD) score at specified intervals. Thus, the tendency to respond to PI treatment with changes in fat metabolism can be genetically mapped. These methods and procedures are well known in the art and have been proven to be efficient in mapping and isolating obesity-linked genes. These same or similar procedures can therefore be used to map

and isolate genes involved in susceptibility to protease inhibitor-induced detrimental effects, such as fat redistribution and/or dyslipidemia.

This invention therefore encompasses the use of the animal model described above to identify and isolate genes associated with protease inhibitor-induced or associated detrimental effects. One skilled in the art will appreciate that using the metabolic effects, the phenotypic effects, and the genotypic effects not previously known but described above, one can now use mice that demonstrate differences in fat metabolism upon treatment with PIs relative to control mice to identify a gene or genes associated with PI-associated detrimental effects. Genes isolated using these procedures can then be used as targets for therapeutic procedures such as inhibition of specific gene expression, stimulation of specific gene expression, and/or to possibly supplement existing gene expression using introduced genes and/or gene fragments, and/or other elements that can be used to control or regulate the expression of these genes and/or other genetic elements that may influence the expression of these genes. Similarly, the genes isolated using these procedures can then be used as targets for diagnostic procedures or themselves used in diagnostic procedures such as immunoassay procedures, nucleic acid detection procedures, detection or classifying the capacity of a patient for a specific protease inhibitor-induced response, and/or nucleic acid amplification procedures. These methods are well known in the art and the genes identified and isolated using the disclosed procedures can be integrated into these methods by a skilled artisan.

Example 4: Effect of NRTIs and NNRTIs on Lipid Metabolism In Mice

Experimental Protocols

Age and weight matched male AKR/J mice (Jackson Labs, Bar Harbor, ME) were housed 5 animals/cage at 72° F and 50% relative humidity with a 12 h light and dark cycle. Starting at 7-8 weeks of age the animals (15 animals/group) were treated with either 5 or 50 mg/kg (PO/bid) and treatment continued for 2 weeks. At the end of the dosing period, the animals were anesthetized with isofluorane, blood drawn by

cardiac puncture, and non-fasting measurements of glucose, total cholesterol, triglycerides, non-esterified free fatty acids (NEFAs), glycerol, alkaline phosphatase, and β-hydroxybutyric acid were obtained. All blood chemistry tests were determined as previously described. Body weight, liver weight and weights of fat depots were determined at the end of the study.

This research complied with the principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and Glaxo Wellcome company policy on the care and use of animals and related codes of practice.

Drugs and Materials: The NRTIs and NNRTIs used in these studies were obtained from the Medicinal Chemistry Department at GlaxoWellcome Inc. (Research Triangle Park, North Carolina).

Data Analysis: The data were calculated as the mean and standard error (SEM) from experiments performed on 15 animals per treatment group. Two-tailed tests were performed to calculate P values using Microsoft Excel.

Results and Discussion

The goal of this study was to determine the effects of HIV RTIs on fat metabolism in AKR/J mice. After dosing at 5 or 50 mg/kg twice a day for 2 weeks, animals (n=15) were sacrificed and lipid metabolism markers were measured.

Table 6 shows the serum measurements of glucose, triglycerides, NEFA and β-HBA in mice treated at 5 mg/kg with EFV, D4T or vehicle. EFV has a very moderate effect: EFV slightly elevates serum triglyceride (p=0.0242) and slightly reduces serum glucose (p=0.0154). D4T causes a strong increase in serum β-HBA (p=0.000015), but no other significant change.

Table 7 shows the results after treatment at 50 mg/kg with EFV, D4T, and vehicle. At this dose, all drugs have a significant effect on serum triglyceride (p-value from 0.000038 to 0.0048) and NEFA (p-value from 0.00000098 to 0.0047). D4T also significantly increases serum β-HBA. EFV also significantly increases serum levels of cholesterol and HDL-cholesterol.

Animal weights, liver weights and fat depot weights were determined at

sacrifice after the 2 week course of treatment (Table 8). The strongest effect was an increase in the liver weight/body weight ratio after high dose treatment with EFV (p=0.000018). Reduction in the SQ fat depot was also observed after high dose treatment with EFV.

In summary, EFV only moderately alters lipid metabolism in AKR/J mice when given at a low dose, while the NRTI D4T has a somewhat stronger effect. At higher doses all drugs studied here significantly elevate triglyceride and NEFA in serum. EFV at higher dose also strongly elevates the liver weight/body weight ratio.

Example 5: Effect of NNRTI Treatment on Gene Expression in Liver and Fat Tissue and on Serum Lipid Levels

METHODS: Age and weight matched male AKR/J mice (Jackson Labs, Bar Harbor, ME) were housed 5 animals/cage at 72° F and 50% relative humidity with a 12 hr light and dark cycle. The animals were fed low fat diet (11.6% kilocalories as fat; NIH R&M/Auto 6F-Ovals 5K67, PMI Feeds® Inc., Richmond, Indiana) throughout the study. Animals were gavaged orally twice daily with vehicle (0.5% HPMC, 310 g/L sucrose), 5 mg/kg efavirenz in vehicle, or 50 mg/kg efavirenz in vehicle. The clinically relevant dose for efavirenz is 600 mg/d. Based on background studies of the effects of multiple pharmacological agents on metabolism in mice, we found two weeks to be sufficient time to allow for significant changes in the blood chemistry tests reported in this study. After two weeks the animals were anesthetized with isofluorane, blood drawn by cardiac puncture, and non-fasting serum measurements given in the tables were made using standard techniques. The data were calculated as the mean and standard error (SEM) from experiments performed on fifteen animals per treatment group. Two-tailed tests were performed to calculate P values. This research complied with the principles of laboratory animal care (NIH publication No. 85-23, revised 1985).

Total RNA was isolated using Qiagen Rneasy kits and quantitated with the RiboGreen reagent (Molecular Probes). TaqMan probes and primers were designed to

match GenBank sequences and ordered from Keystone Labs. RT-PCR was performed in the following reaction mix: standard TaqMan buffer conditions (PE Biosystems) with 300nm primers, 100nm probe, and 25 or 125 ng total RNA. The RT reaction was performed at 48 C for 30 minutes, followed by standard cycling conditions on the 7700 Sequence Detector (PE Biosystems). All samples were assayed in duplicate, and there were 6 samples per group (vehicle vs. Efavirenz). Results were initially obtained as the cycle number at which the fluorescence had increased 10-fold from initiation of the reactions. Lower cycle numbers reflect greater gene expression. Results from each group were averaged, and then compared to each other to provide a p-value. Fold-differences were calculated from the averages.

RESULTS: Efavirenz increased FAS expression in fat tissue and decreased FAS expression in liver (see Tables 9 and 10). There was no effect of efavirenz on expression of TNFα in either tissue or HMG CoA synthase in liver (see Tables 9 and 10. Efavirenz inhibited expression of PPARγ, CPT-1, UCP-2, and DGAT in liver (see Table 10. Efavirenz treatment also significantly increased liver but not fat mass in mice (see Table 11). As expected, serum alkaline phosphatase increased in parallel to hepatomegaly. Serum triglycerides, fatty acids, and cholesterol levels increased after efavirenz treatment. Efavirenz had no adverse effects on serum glucose or ketone body levels (Table 11).

Throughout this application, various publications are referenced. The disclosures of these publications, and the references cited therein, in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and example be considered as

exemplary only, with a true scope and spirit of the invention being indicated by the claims.

Table 1. Effects of PIs and diet on insulin resistance

	Insulin (pmol/L)	Glucose (mmol/L)	IR Index (Insulin x Glucose)
LOW FAT			
Placebo	182.2 ± 36.4	10.8 ± 0.4	1967.8 ± 323.2
APV	139.1 ± 36.4 (0.2)	10.1 ± 0.3 (0.07)	1404.9 ± 382.3 (0.12)
SQV	112.6 ± 16.6 (0.032)	10.4 ± 0.3 (0.19)	1171.0 ± 175.2 (0.019)
IDV	89.4 ± 11.6 (0.01)	11.9 ± 0.4 (0.02)	1063.9 ± 146.7 (0.011)
NLV	87.8 ± 18.2 (0.003)	12.4 ± 0.6 (0.016)	1088.7 ± 162.3 (0.027)
HIGH FAT			
Placebo	336.2 ± 145.8	13.3 ± 0.7	4471.5 ± 1891.5
APV	294.8 ± 89.4 (0.4)	13.4 ± 1.0 (0.46)	3950.3 ±1353.3 (0.43)
SQV	327.9 ± 82.8 (0.47)	13.3 ± 0.6 (0.46)	4361.1 ± 1088.1 (0.47)
IDV	303.1 ± 66.3 (0.4)	12.4 ± 0.3 (0.14)	3758.4 ± 774.1 (0.35)
NLV	261.7 ± 59.6 (0.33)	13.5 ± 0.6 (0.38)	3532.9 ± 1021.1 (0.36)

IR, insulin resistance; Numbers in the parenthesis denote the statistical significance (P value) of the experimental groups relative to the placebo groups calculated using Student's t test.

Table 2. Effects of PIs and diet on serum lipids and lipase

	Triglycerides (mmol/L)	NEFAs (mmol/L)	Glycerol (mmol/L)	Cholesterol (mmol/L)	Lipase (U/L)
LOW FAT					
Placebo	1.91 ± 0.09	1.07 ± 0.09	2.25 ± 0.31	1.59 ± 0.04	76 ± 2
APV	1.57 ± 0.11 (0.012)	1.19 ± 0.56 (0.13)	3.06 ± 0.28 (0.029)	1.66 ± 0.04 (0.08)	79 ± 4 (0.22)
SQV	1.51 ± 0.09 (0.0016)	1.09 ± 0.75 (0.42)	2.45 ± 0.20 (0.28)	1.57 ± 0.08 (0.39)	75 ± 2 (0.32)
IDV	1.81 ± 0.17 (0.28)	1.33 ± 0.10 (0.034)	3.11 ± 0.42 (0.047)	1.63 ± 0.05 (0.25)	83 ± 2 (0.003)
NLV	1.76 ± 0.07 (0.07)	1.43 ± 0.09 (0.005)	3.50 ± 0.34 (0.005)	1.63 ± 0.05 (0.27)	83 ± 3 (0.031)
HIGH FAT					
Placebo	1.73 ± 0.18	1.38 ± 0.20	3.99 ± 0.63	2.59 ± 0.05	83 ± 2
APV	1.85 ± 0.17 (0.32)	1.42 ± 0.08 (0.43)	3.81 ± 0.21 (0.38)	2.59 ± 0.08 (0.45)	86 ± 23 (0.15)
SQV	2.17 ± 0.10 (0.023)	1.46 ± 0.04 (0.3)	3.98 ± 0.15 (0.49)	2.83 ± 0.08 (0.014)	89 ± 1 (0.007)
IDV	2.76 ± 0.20 (0.0007)	1.71 ± 0.07 (0.04)	5.02 ± 0.24 (0.05)	2.70 ± 0.05 (0.05)	101 ± 4 (0.0002)
NLV	2.57 ± 0.14 (0.001)	1.72 ± 0.08 (0.05)	5.04 ± 0.39 (0.07)	2.65 ± 0.08 (0.28)	89 ± 4 (0.09)

NEFAs, non-esterified fatty acids. Numbers in the parenthesis denote the statistical significance (P value) of the experimental groups relative to the placebo groups calculated using Student's t test.

Table 3. Plasma health screens

	ALP (U/L)	BILRUBIN (μmol/L)	ALT (U/L)	LDH (U/L)	BUN (mmol/L)
LOW FAT					
Placebo	69.6 ± 3.6	22.04 ± 0.86	38 ± 4	216 ± 21	10.55 ± 0.46
APV	75.1 ± 4.1 (0.15)	22.55 ± 0.82 (0.27)	51 ± 12 (0.12)	354 ± 61 (0.015)	9.62 ± 0.29 (0.045)
SQV	74.7 ± 4.8 (0.18)	23.23 ± 0.86 (0.12)	57 ± 19 (0.15)	287 ± 57 (0.11)	9.87 ± 0.50 (0.14)
IDV	86.7 ± 3.2 (0.0009)	24.43 ± 0.86 (0.017)	207 ± 130 (0.09)	559 ± 261 (0.09)	9.41 ± 0.53 (0.048)
NLV	84.7 ± 4.8 (0.008)	24.26 ± 0.34 (0.008)	202 ± 109 (0.05)	437 ± 184 (0.07)	9.08 ± 0.50 (0.017)
HIGH FAT					
Placebo	58 ± 1.6	23.23 ± 1.37	142 ± 52	345 ± 145	7.23 ± 0.29
APV	54.3 ± 1.7 (0.056)	23.06 ± 1.37 (0.45)	66 ± 17 (0.09)	384 ± 69 (0.45)	7.94 ± 0.32 (0.38)
SQV	55.2 ± 1.8 (0.12)	25.80 ± 1.37 (0.014)	54 ± 17 (0.05)	238 ± 48 (0.2)	6.91 ± 0.11 (0.49)
IDV	66.8 ± 1.8 (0.0007)	33.31 ± 1.20 (0.05)	142 ± 59 (0.49)	473 ± 143 (0.26)	9.65 ± 0.36 (0.05)
NLV	63.2 ± 3.1 (0.059)	28.36 ± 0.85 (0.28)	153 ± 90 (0.46)	628 ± 278 (0.18)	9.16 ± 0.32 (0.07)

ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; Numbers in the parenthesis are P values calculated using Student's t test and denote the statistical significance between the experimental and the placebo group.

Table 4. Effects of PIs and diet on fat mass

	Intrascapular	Epididymal
LOW FAT		
Placebo	0.0642 ± 0.0072	0.093 ± 0.012
APV	0.0717 ± 0.0072 (0.22)	0.131 ± 0.022 (0.11)
SQV	0.0824 ± 0.0078 (0.04)	0.131 ± 0.011 (0.02)
IDV	0.0765 ± 0.0052 (0.08)	0.138 ± 0.019 (0.04)
NLV	0.0845 ± 0.0067 (0.002)	0.121 ± 0.018 (0.18)
HIGH FAT		
Placebo	0.1127 ± 0.0138	0.3962 ± 0.0302
APV	0.0981 ± 0.0111 (0.20)	0.3495 ± 0.0438 (0.18)
SQV	0.0962 ± 0.0075 (0.14)	0.4011 ± 0.0241 (0.45)
IDV	0.1015 ± 0.0101 (0.24)	0.4261 ± 0.0232 (0.21)
NLV	0.939 ± 0.0067 (0.10)	0.3861 ± 0.041 (0.42)

Normalized data was calculated by determining the ratio of fat mass to liver mass for each treatment group. Numbers in the parenthesis denote the statistical significance (P value) of the experimental groups relative to the placebo groups calculated using Student=s t test.

Table 5. Plasma drug concentrations (ng/ml)

	Low Fat	High Fat	P-Value
APV	24.1 ± 3.3	33.1 ± 0.5	0.09
SQV	71.4 ± 16.7	28.4 ± 0.6	0.015
IDV	13.4 ± 1.4	16.7 ± 2.5	0.54

NLV	4.1 ± 0.4	4.1 ± 0.6	0.94

Table 6. Serum markers of lipid metabolism: low dose RTIs (5 mg/kg)

		Glucose mg/dL	Triglycerides mg/dL	NEFA mEq/L	β-HBA mg/dL
Vehicle	Mean (SEM)	259 (10.3)	173 (6.6)	0.74 (0.04)	2.02 (0.11)
EFV	Mean (SEM)	230 (8.2)	196 (9.4)	0.82 (0.06)	2.14 (0.09)
	p-value	0.0154	0.0242	0.1304	0.2149
D4T	Mean (SEM)	242 (7.2)	190 (9.0)	0.65 (0.04)	2.76 (0.10)
	p-value	0.0881	0.0599	0.0522	0.000015

Table 7. Serum markers of lipid metabolism: high dose RTIs (50 mg/kg)

		Glucose mg/dL	Triglycerides mg/dL	NEFA mEq/L	β-HBA mg/dL
Vehicle	Mean (SEM)	211 (10.9)	144 (9.8)	0.90 (0.04)	1.67 (0.13)
EFV	Mean (SEM)	195 (11.9)	186 (12.0)	1.13 (0.04)	1.75 (0.14)
	P-value	0.1494	0.00484	0.0003	0.3219
D4T	Mean (SEM)	195 (10.6)	211 (11.0)	1.23 (0.03)	1.32 (0.13)
	P-value	0.1359	0.000038	0.00000098	0.0282

Table 8. Body, Liver and Fat Depot Weights after high dose RTIs (50 mg/kg)

	Delta BW's	LIV/BW (X100)	Fat Depot SQ/BW (X1000)	Fat Depot EPI/BW (X1000)	Fat Depot BAT/BW (X1000)	Fat Depot MES/BW (X1000)	Fat Depot REN/BW (X1000)
Veh	**0.73**	**5.26**	**2.15**	**8.87**	**4.31**	**5.01**	**2.87**
SEM	0.513	0.121	0.201	0.637	0.312	0.797	0.328
Efavirenz	**0.79**	**6.40**	**1.42**	**8.53**	**3.78**	**4.20**	**2.66**
SEM	0.347	0.211	0.152	1.033	0.262	0.668	0.307
p value	0.46	1.8E-05	0.004	0.38	0.099	0.22	0.32
D4T	**0.68**	**5.51**	**2.10**	**8.51**	**4.11**	**4.49**	**2.60**
SEM	0.472	0.160	0.202	0.921	0.381	0.711	0.300
p value	0.47	0.10	0.43	0.37	0.24	0.31	0.27

Table 9:

Gene	Control	Efavirenz	P-value	Fold-Change
FAS	21.57 ± 0.35	19.62 ± 0.30	<0.05	3.9X increase
TNFα	25.07 ± 0.53	25.06 ± 0.33		

FAS, Fatty acid Synthase; TNFα, Tumor necrosis factor alpha. Threshold data are means ± SEM. For PCR, lower numbers reflect greater gene expression. Two-tailed student's tests were performed to calculate P values.

Table 10:

Gene	Control	Efavirenz	P-value	Fold-Change
FAS	22.18 ± 0.29	22.88 ± 0.20	0.05	1.62X decrease
PPARα	19.14 ± 0.13	20.19 ± 0.22	0.001	2.07X decrease
UCP-2	22.09 ± 0.27	23.21 ± 0.12	0.002	2.17X decrease
CPT-1	18.08 ± 0.11	18.52 ± 0.18	0.044	1.66X decrease
DGAT	21.03 ± 0.12	21.79 ± 0.16	0.002	1.69X decrease
TNFα	26.64 ± 0.53	27.17 ± 0.26	>0.05	
HMG CoA S	16.20 ± 0.07	16.48 ± 0.13	>0.05	

FAS, Fatty acid Synthase; PPAR, peroxisome proliferator activated receptor; UCP, uncoupling protein; CPT, carnitine palmitoyl transferase; DGAT, diacylglycerol acyl transferase; TNFα, Tumor necrosis factor alpha. HMG CoA S, 3-hydroxy-3-methylglutaryl-synthase. Threshold data are means ± SEM. For PCR, lower numbers reflect greater gene expression. Two-tailed student's tests were performed to calculate P values.

Table 11:

Assay (units)	Control	Efavirenz	P-value
Alkaline phosphatase (U/L)	94 ± 4.3	18 ± 5	0.001
Cholesterol (mg/dL)	75 ± 2.0	83 ± 2.4	0.0064
Free fatty acids (mEq/L)	0.9 ± 0.04	1.13 ± 0.04	0.0006
Glucose (mg/dL)	211 ± 11	195 ± 12	0.3
Glycerol (mg/dL	21 ± 1.5	19 ± 1.6	0.54
HDL-cholesterol (mg/dL)	52 ± 1.3	55 ± 1.4	0.056
β-Hydroxybutyric acid (mg/dL)	1.67 ± 0.13	1.75 ± 0.14	0.64
Triglycerides (mg/dL)	144 ± 9.8	186 ± 12.3	0.0096
Liver Mass (% of body wt)	5.26 ± 0.12	6.4 ± 0.21	0.00004
Interscapular Fat mass	0.43 ± 0.03	0.38 ± 0.03	0.18
Epididymal Fat Mass	0.89 ± 0.06	0.85 ± 0.1	0.76
Change in body weight (g)	0.7 ± 0.5	0.8 ± 0.3	0.92

Data are means ± SEM. Two-tailed student's tests were performed to calculate P values.

What is claimed is:

1. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising

(a) administering the RTA to a mesenchymal stem cell or pre-adipocyte cell under culture conditions appropriate for adipogenesis; and

(b) monitoring the cell for an inhibition of adipogenesis;

whereby inhibition of adipogenesis indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in the patient.

2. The method of claim 1, wherein the RTA is administered to a mesenchymal stem cell.

3. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

(a) administering the RTA to a cell capable of metabolizing lipids under conditions permissible for lipogenesis; and

monitoring net lipogenesis in the cell, whereby a change in net lipogenesis in the cell indicates the protease inhibitor can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient.

4. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

(a) administering the RTA to a cell capable of metabolizing lipids under conditions permissible for lipolysis; and

(b) monitoring net lipolysis in the cell, whereby a change in net lipolysis in the cell indicates the protease inhibitor can affect lipodystrophy or

dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient

5. The method of any of claims 3 or 4, wherein the cell to which the RTA is administered is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

6. The method of any of claims 1, 3 or 4, wherein the RTA is a protease inhibitor.

7. The method of any of claims 1, 3 or 4, wherein the RTA is a NRTI.

8. The method of any of claims 1, 3 or 4, wherein the culture conditions comprise culturing the cell in the presence of a receptor ligand selected from the group consisting of a PPARγ ligand, a RXR ligand, a retinoic acid receptor ligand, insulin, an insulin-like growth factor, a glucocorticoid receptor ligand, and a cAMP-elevating agent.

9. The method of claim 8, wherein the receptor ligand is a PPARγ ligand.

10. The method of claim 9, wherein the PPARγ ligand is an agonist of PPARγ.

11. The method of claim 10, wherein the PPARγ agonist is a thiazolidinedione.

12. The method of claim 8, wherein the receptor ligand is a RXR ligand.

13. The method of claim 12, wherein the RXR ligand is an agonist of RXR.

14. The method of claim 13, wherein the RXR agonist is LGD1069, LG100268, 9-cis retinoic acid, or all-trans retinoic acid.

15. The method of claim 8, wherein the receptor ligand is a retinoic acid receptor ligand.

16. The method of claim 15, wherein the retinoic acid ligand is CH55, 9-cis retinoic acid, or all-trans retinoic acid.

17. The method of claim 8, wherein the receptor ligand is insulin.

18. The method of claim 8, wherein the receptor ligand is an insulin-like growth factor.

19. The method of claim 6, wherein the protease inhibitor is an aspartyl protease inhibitor.

20. The method of claim 19, wherein the aspartyl protease inhibitor is a viral aspartyl protease inhibitor.

21. The method of claim 20, wherein the viral aspartyl protease inhibitor is an HIV protease inhibitor.

22. The method of claim 7, wherein the NRTI is an HIV NRTI.

23. The method of any of claims 2 or 5, wherein the mesenchymal stem cell has the characteristics of a C3H10T1/2 cell.

24. The method of claim 23, wherein the mesenchymal stem cell is a mammalian primary cell.

25. The method of claim 24, wherein the mammalian primary cell is a human primary cell.

26. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:

(a) administering the RTA to a cell capable of metabolizing lipids under conditions permissible for metabolizing lipids; and

(b) monitoring the expression of a PPARγ:RXR-regulated gene in the cell, whereby a change in gene expression of the PPARγ:RXR-regulated gene indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in the patient.

27. The method of claim 26, wherein the cell capable of metabolizing lipids is selected from the group consisting of a mesenchymal stem cell, a liver cell, a muscle cell, an osteoblast, a Schwann cell, an adipocyte, and a pre-adipocyte.

28. The method of claim 26, wherein the RTA is a protease inhibitor.

29. The method of claim 26, wherein the PPARγ:RXR-regulated gene is a gene which encodes aP2.

30. The method of claim 26, wherein the PPARγ:RXR-regulated gene is a gene which encodes lipoprotein lipase.

31. A method of screening a PI for its capacity to affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity in a patient comprising:

(a) administering the PI to a cell containing a retinoid-regulated gene in the presence of a retinoid; and

(b) monitoring the cell for a change in the expression of the retinoid-activated gene, whereby a change in the expression of the retinoid-

activated gene indicates the PI can affect affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity, thereby screening the PI for its capacity to affect affect lipodystrophy, dyslipidemia, or retinoid-associated toxicity in the patient.

32. The method of claim 31, wherein the cell is an adipocyte or a preadipocyte.

33. The method of claim 31, wherein the PI is an HIV PI.

34. The method of claim 31, wherein the retinoid-activated gene is a gene which encodes alkaline phosphatase.

35. A method of screening a compound for its potential to effect fat metabolism comprising:

(a) contacting a PPARγ receptor-ligand complex with the compound; and

(b) monitoring the complex for displacement of the receptor ligand from the complex, whereby a compound that displaces the receptor has a potential to effect fat metabolism, thereby screening the compound for its potential to effect fat metabolism.

36. A method of screening a compound for its potential to effect fat metabolism comprising:

(a) contacting a PPARγ receptor-ligand complex with the compound; and

(b) monitoring the complex for binding of the compound to the complex, whereby a compound that binds to the complex receptor has a potential to effect fat metabolism, thereby screening the compound for its potential to effect fat metabolism.

37. The method of claim 35 or claim 36, wherein the compound is screened for potential protease inhibitor activity.

38. The method of claim 35 or claim 36, wherein the receptor ligand is a PPARγ ligand.

39. The method of claim 38 wherein the PPARγ ligand is a thiazolidinedione.

40. The method of claim 38, wherein the ligand is BRL49653.

41. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising:
(a) administering the RTA to a mammal susceptible to diet-induced obesity; and
(b) monitoring the mammal for an increase in serum lipids, whereby the increase in net serum lipids indicates the RTA has the capacity to increase lipodystrophy or dyslipidemia in a patient.

42. The method of claim 41, wherein the change in serum lipids is indicated by a change in serum triglycerides, free fatty acids, glycerol, or cholesterol.

43. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient comprising:
a) administering the RTA to a mammal susceptible to diet-induced obesity; and
b) monitoring net fat deposition in the mammal, whereby a change in net fat deposition indicates the RTA can affect lipodystrophy or dyslipidemia, thereby screening the RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient.

44. The method of claim 43, wherein the change in net fat deposition is indicated by

a change in the weight of fat pads.

45. The method of claim 43, wherein the change in net fat deposition is indicated by a change in expression or activity of proteins produced by adipocytes.

46. The method of claim 43, wherein the fat deposition results in interscapular or epididymal fat depots.

47. A method of screening an RTA for its capacity to affect lipodystrophy or dyslipidemia in a patient, comprising:

(a) administering the RTA to a mammal susceptible to diet-induced obesity; and

(b) monitoring the mammal for an increase in blood urea nitrogen or glucose, whereby the increase in blood urea nitrogen or glucose indicates the RTA has the capacity to affect lipodystrophy or dyslipidemia in a patient.

48. A method of screening an RTA for its capacity to affect lipodystrophy, dyslipidemia or retinoid associated toxicities in a patient, comprising:

(a) administering the RTA to a cell containing a retinoid-regulated gene in the presence of a retinoid; and

(a) monitoring the mammal for a change in the expression of a retinoid-activated gene, whereby a change in the expression of the retinoid-activated gene indicates the RTA can affect lipodystrophy, dyslipidemia, or retinoid associated toxicities, thereby screening the RTA for its capacity to affect lipodystrophy, dyslipidemia, or retinoid associated toxicities in a patient.

49. The method of any of claims 41, 43, 47, or 48, wherein the RTA is a protease

inhibitor.

50. The method of any of claims 41, 43, 47, or 48, wherein the mammal is maintained under high-fat diet conditions.

51. The method of any of claims 41, 43, 47, 48, 0r 58 wherein the mammal is a mouse.

52. The method of claim 41, 43, 47, 48, or 58, wherein the mouse has the obesity-related characteristics of a AKR/J mouse.

53. The method of claim 48, wherein the retinoid-activated gene is a gene which encodes alkaline phosphatase.

54. The method of claim 48, wherein the retinoid-activated gene is activated by a retinoid nuclear receptor.

55. A transgenic mouse whose somatic cells comprise and express a transgene conferring sensitivity to an RTA, wherein the total native and transgene expressed in the transgenic mouse is higher than the native gene expressed in a non-transgenic mouse, which transgenic mouse has a phenotype of increased sensitivity to the RTA.

56. A transgenic mouse whose somatic cells comprise and overexpress ubiquitously in all tissues a transgene conferring sensitivity to an RTA, wherein the total native and transgene expressed in the transgenic mouse is higher than the native gene expressed in a non-transgenic mouse, which transgenic mouse has a phenotype of increased sensitivity to the RTA.

57. The transgenic mouse of claim 55 or 56, wherein the RTA is a protease inhibitor.

58. A method of identifying a compound for treating RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to an RTA-sensitive mouse, and monitoring the mouse for a change in the expression of a gene and/or the activity of a gene product associated with lipodystrophy or dyslipidemia, a change in fat distribution, and/or a change in serum lipids, whereby a change in the change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to decrease lipodystrophy or dyslipidemia in the mammal and thereby treat RTA-induced lipodystrophy or dyslipidemia in a mammal.

59. The method of claim 58, wherein the RTA is a HIV protease inhibitor, HIV NRTI or HIV NNRTI.

60. A method of detecting a capacity of a compound to cause RTA-induced lipodystrophy or dyslipidemia in a mammal, comprising administering the compound to an RTA-sensitive mouse, monitoring the mouse for a change in expression of a gene and/or the activity of a gene product associated with lipodystrophy, dyslipidemia or retinoid associated toxicities in the mouse, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and/or the activity of the gene product, an increase in fat distribution, or a decrease in serum lipids indicates the compound has the capacity to cause RTA-induced lipodystrophy, dyslipidemia or retinoid associated toxicities in the mammal.

61. The method of claim 60, wherein the RTA is a protease inhibitor.

62. A method of classifying a patient as being susceptible to RTA-induced lipodystrophy or dyslipidemia, comprising administering RTA to the patient, monitoring the patient for a change in the expression of a gene and/or the activity of a gene associated with lipodystrophy, dyslipidemia or retinoid associated toxicities, a change in fat distribution, and/or a change in serum lipids, whereby a change in the expression of the gene and or the activity of the gene product, an increase in fat distribution, and/or a decrease in serum lipids indicates the patient may be susceptible to lipodystrophy or dyslipidemia; thereby classifying the patient as being susceptible to RTA-induced lipodystrophy, dyslipidemia or retinoid associated toxicities.

63. The method of claim 62, wherein the RTA is a HIV protease inhibitor, HIV NRTI, HIV, NNRTI.

64. The method of any of claims 58, 60, or 62, wherein the RTA is an HIV protease inhibitor.

65. The method of any of claims 58, 60, or 62, wherein the gene is a retinoid-activated gene.

66. The method of any of claims 58, 60, or 62, wherein the gene is activated by a retinoid nuclear receptor.

67. The method of any of claims 58, 60, or 62, wherein the gene is a PPARγ:RXR-activated gene.

68. The method of any of claims 58, 60, or 62, wherein the gene is a protease inhibitor regulated gene.

69. The method of any of claims 58, 60, or 62, wherein the change in gene expression comprises an increase in gene expression.

70. The method of any of claims 58, 60, or 62, wherein the change in gene expression comprises a decrease in gene expression.

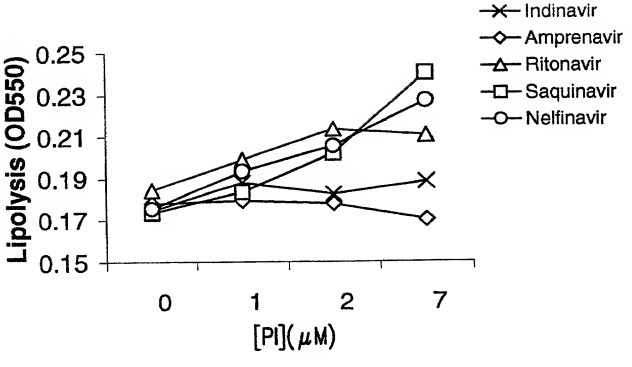
Indinavir
Amprenavir
Ritonavir
Saquinavir
Nelfinavir
Lipolysis (OD550)
0.25
0.23
0.21
0.19
0.17
0.15
0
1
2
7
[PI](μM)

FIG. 1

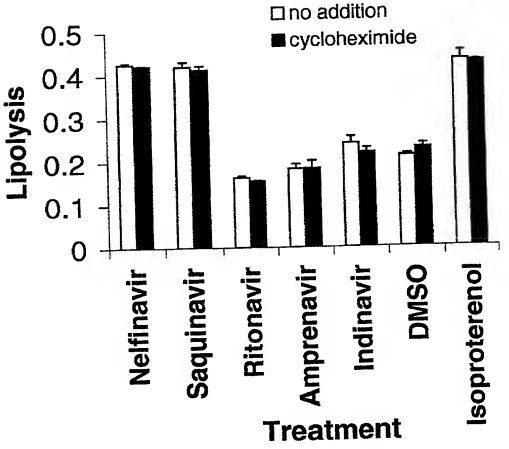
no addition
cycloheximide
Lipolysis
0.5
0.4
0.3
0.2
0.1
0
Nelfinavir
Saquinavir
Ritonavir
Amprenavir
Indinavir
DMSO
Isoproterenol
Treatment

FIG.2

PROTEASE INHIBITOR	IC50 (μM)
NELFINAVIR	8.7 $\pm$ 1.7
SAQUINAVIR	9.7 $\pm$ 6.5
RITONAVIR	17.0 $\pm$ 2.7
INDINAVIR	>20.0
AMPRENAVIR	>20.0

FIG.3

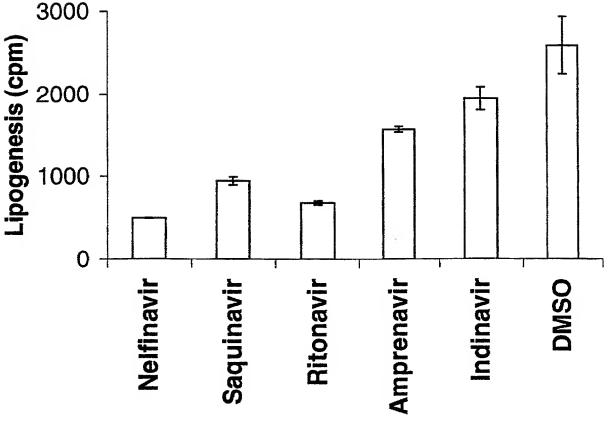
Lipogenesis (cpm)
3000
2000
1000
0
Nelfinavir
Saquinavir
Ritonavir
Amprenavir
Indinavir
DMSO

FIG. 4

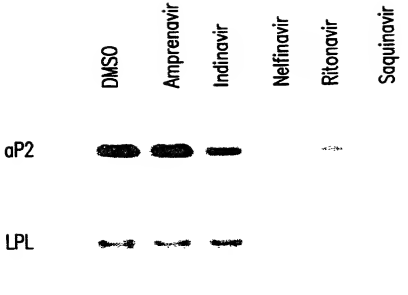
DMSO
Amprenavir
Indinavir
Nelfinavir
Ritonavir
Saquinavir
aP2
LPL

FIG.5

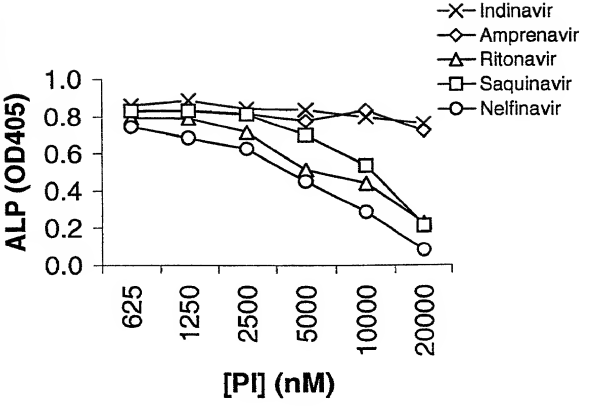
Indinavir
Amprenavir
Ritonavir
Saquinavir
Nelfinavir
ALP (OD405)
1.0
0.8
0.6
0.4
0.2
0.0
625
1250
2500
5000
10000
20000
[PI] (nM)

FIG. 6

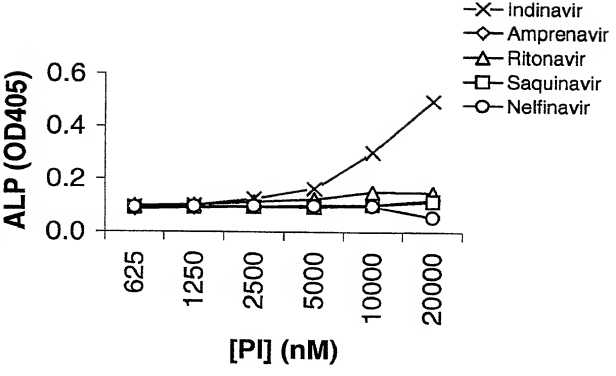
Indinavir
Amprenavir
Ritonavir
Saquinavir
Nelfinavir
ALP (OD405)
0.6
0.4
0.2
0.0
625
1250
2500
5000
10000
20000
[PI] (nM)

FIG. 7

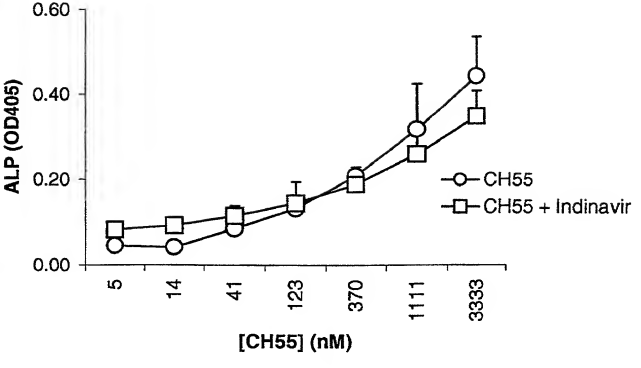
0.60
0.40
0.20
0.00
ALP (OD405)
5
14
41
123
370
1111
3333
[CH55] (nM)
CH55
CH55 + Indinavir

FIG. 8

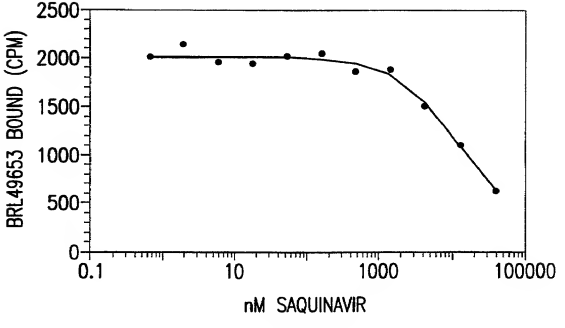
BRL49653 BOUND (CPM)
2500
2000
1500
1000
500
0
0.1
10
1000
100000
nM SAQUINAVIR

FIG.9

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e))

Attorney Docket Number	PU3610USW
First Named Inventor	James Martin Lenhard
COMPLETE IF KNOWN	
Application Number	/
Filing Date	
Group Art Unit	
Examiner Name	

23347
PATENT TRADEMARK OFFICE

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS OF SCREENING PROTEASE INHIBITORS, OF INDUCING MICE SUSCEPTIBLE TO HIV PROTEASE INHIBITOR-INDUCED DYSLIPIDEMIA, AND GENES ASSOCIATED THEREWITH

the specification of which (*Title of the Invention*)

☐ is attached hereto
OR
☒ was filed on (MM/DD/YYYY) [ ] as United States Application Number or PCT International Application Number PCT/US00/01205 and was amended on (MM/DD/YYYY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above:

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign		Foreign Filing Date	Priority	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/116,300	01/19/1999
60/137,620	06/04/1999
60/146,309	07/27/1999

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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Application Number	
Filing Date	
First Named Inventor	James Martin Lenhard
Group Art Unit	
Examiner Name	
Attorney Docket Number	PU3610USW

I hereby appoint:

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I am the:

☒ Applicant/Inventor.

☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

SIGNATURE of Applicant or Assignee of Record

Name	James Martin Lenhard
Signature	James Martin Lenhard
Date	7/10/01

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☒ *Total of 1 forms are submitted.

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DECLARATION — Utility or Design Patent Application

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Address			
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City		State	ZIP
Country	Telephone		Fax

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NAME OF SOLE OR FIRST INVENTOR :		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any]) James Martin		Family Name or Surname LENHARD	
Inventor's Signature James Martin Lenhard			Date 7/10/01
Residence: City Durham NC	State NC	Country US	Citizenship US
Mailing Address GlaxoSmithKline			
Mailing Address Five Moore Drive, PO Box 13398			
City Research Triangle Park	State NC	ZIP 27709	Country US

NAME OF SECOND INVENTOR:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
Inventor's Signature			Date
Residence: City	State	Country	Citizenship
Mailing Address			
Mailing Address			
City	State	ZIP	Country

☐ Additional inventors are being named on _____ supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.